0001

Vascular endothelial growth factor production from epidermal keratinocytes is enhanced by vasoactive intestinal peptide and cytokines

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Vascular endothelial growth factor (VEGF) can induce angiogenesis. VEGF has been implicated in the angiogenesis observed in psoriatic lesion. Recent evidences suggest an active role of vasoactive intestinal peptide (VIP) in the pathogenesis of inflammatory skin disease such as psoriasis. The aim of this study is to determine whether VIP and cytokines (IL-4/IFN-γ/TNF-α) can induce the production of VEGF from keratinocytes. DJM-1 cells (human epidermal keratinocyte cell line) were incubated for 6 h in the presence of VIP (10μM) and/or cytokines (1ng/ml). The concentrations of VEGF in the supernatants were assessed by a specific enzyme-linked immunosorbent. VEGF from VIP-and /or cytokines-treated DJM-1 cell were significantly increased compared to the control. The combination of VIP and IFN-γ synergistically enhanced VEGF from DJM-1 cells.These results suggest that several inflammatory cytokines and VIP from mast cells and nerve ending are capable of inducing VEGF production from keratinocytes, resulting in the angiogenesis of inflammatory dermatoses such as psoriasis.

0002

Normal epithelial cells modulate capillary size in a human *in vitro* angiogenesis model produced by the self-assembly method

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Epithelial cells can modulate the vascularization of their underlying stroma according to their metabolic needs by secreting a multitude of soluble factors to modulate the properties of the nearby capillaries. To determine the importance of epithelial cells on the organization of endothelial cells into capillaries, a human self-assembled cutaneous angiogenesis model was used. This *in vitro* angiogenesis model is devoid of exogenous or animal extracellular matrix components, and contains a dynamic tridimensionnal capillary network responsible to external stimuli. When keratinocytes were seeded on the surface of our *in vitro* model of angiogenesis, the capillaries were small (area covered less than 50 μm^2), ressembling those found in the human skin. On the other hand, when capillaries were grown in the absence of epithelial cells, they then formed bigger capillaries (area covered more than 50 μm^2). The size of these giant capillaries can be reduced by the addition of epithelial cells (skin, cornea, uterus) on our *in vitro* model of angiogenesis. The formation of the giant capillaries in our *in vitro* model devoid of keratinocytes can also be prevented by the addition of keratinocyte's conditionned media. These results demonstrate that keratinocytes do not have to be in direct contact with the reconstructed stroma to modulate capillary size. Furthermore, such results also emphasize the importance of soluble factors produced by keratinocytes in the homeostasis of the microcirculation's vessels.

0003

The bicyclic monoterpene diol AGI-1140 induces angiogenic factors

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Angiogenesis is an important step in wound healing in skin. The bicyclic monoterpene diol AGI-1140 (2,2-dimethyl-3-propanyldiol-norbornane) has been shown to induce nitric oxide (NO) production in mouse melanoma cells and since NO production is an inducer of angiogenesis, we examined the ability of AGI-1140 to induce molecular markers of angiogenesis. AGI-1140 induced NO production in both human umbilical vein and microvascular endothelial cells in a dose-dependent manner. Treatment of endothelial cells with 500 μM AGI-1140 caused an increase in cellular invasion through a reconstituted basement membrane similar to the increase produced by 100 ng/ml vascular endothelial growth factor (VEGF), an angiogenic factor now in clinical testing. Zymographic analysis revealed that both AGI-1140 and VEGF increases matrix metalloproteinase-2 (MMP-2; Gelantinase A) secretion from endothelial cells. The addition of 1 ng/ml transforming growth factor β (TGF-β) to AGI-1140 further increases MMP-2 secretion, while it did not increase the secretion induced by VEGF. ELISA analysis revealed that VEGF caused a substantial increase in the expression of platelet derived growth factor (PDGF-BB), while AGI-1140 only slightly increased PDGF-BB expression. However, AGI-1140 in combination with TGF-β increased the expression of PDGF-BB synergistically, while the combination of VEGF and TGF-β did not further increase PDGF-BB expression above that of VEGF alone. VEGF expression was not affected by AGI-1140 as measured by ELISA. Therefore, AGI-1140 alone stimulates some, but not all, molecular markers of angiogenesis.

0004

Vascular endothelial growth factor receptor 2 gene expression by endothelial cells is inhibited by PPARα agonists but not by PPARγ agonists

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, mainly implicated in the regulation of lipid and glucose homeostasis. In addition, natural and synthetic PPAR agonists may control inflammatory processes by inhibition of distinct proinflammatory genes. As signaling via the vascular endothelial growth factor receptor 2 (VEGFR2) pathway is critical for angiogenic responses during chronic inflammation, we explored whether anti-inflammatory effects of PPAR ligands could be mediated in part through diminished VEGFR2 expression. In this study, PPARα agonists are found (WY-14643, fenofibrate) to potently inhibit endothelial VEGFR2 expression, whereas predominant PPARγ ligands (ciglitazone, 15d-PGJ2) remained without discernable effects. Time- and concentration-dependent inhibition is demonstrated both at the level of protein (FACS, Western blot analysis) and mRNA VEGFR2 expression (Northern blot analysis). Inhibitory effects of PPARα agonists on transcriptional activity of the VEGFR2 promoter is conveyed by an element located between bp −60 and −37 that contains two adjacent consensus Sp1 transcription factor binding sites. Constitutive Sp1-dependent DNA binding to this sequence is decreased by PPARα treatment, indicating that PPARα activation may interfere with Sp1-dependent VEGFR2 transcription. Since PPARα agonists greatly attenuate VEGF-driven capillary-like network formation as VEGFR2-dependent endothelial cell function, anti-inflammatory effects of PPARα ligands may be likely mediated in part via reduced VEGFR2 expression.

0005

The characterization of pericytes with 3G5 antigen in angiogenesis of decubitus skin

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It has been reported the pericyte population is heterogenous in the phenotypical markers and locations of normal and diseased tissues. Although it has been accepted that perycytes express α-SMA or desmin, several reports showed the presence of α-SMA or desmin negative pericytes. 3G5 antigen is expressed on α-SMA negative pericytes in normal skin. Additionally, HMW-MAA has been shown as a activated pericyte marker in tumor stroma and angiogenesis. The purpose of this study is to characterize pericyte in granulation tissues by these markers. Immunohistochemical staining and double immunofluorescence staining were performed on sections from normal and decubitus tissues. 3G5 positive cells, and α-SMA, desmin or HMW-MAA were exclusively present in normal and decubitus tissues. Futhermore, the presence of interstitial 3G5 positive cells without the coexistence of type4 collagen were found. And a part of these cells showed double positive with AS02, which was used as a marker for fibroblast, in the papillary dermis. Our data suggest that the interstitial 3G5 positive cells might be pericyte precursor cells in angiogenesis.

0006

Fibroblast growth factor-2 induces Lef/Tcf-dependent transcription in human endothelial cells

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Lef/Tcf proteins belong to a family of architectural transcription factors, which control developmental processes and play an important role in oncogenesis. Classical activators of Lef/Tcf-dependent transcription comprise the Wnt family of proteins, which translocate beta-catenin into the nucleus and allow the formation of transactivation-competent Lef/Tcf/beta-catenin complexes. Here we show that in human endothelial cells fibroblast growth factor-2 (FGF-2) reduces GSK-3 activity and augments nuclear levels of beta-catenin. FGF-2 induced Lef/Tcf-dependent transcription of a cyclin D1-luciferase construct. Gel shift assays revealed binding of Tcf-4 as the only Lef/Tcf family member and of beta-catenin to the Lef/Tcf site in the cyclin D1 promoter. Cotransfection with a dominant-negative Tcf-4 construct inhibited the FGF-2-induced cyclin D1 promoter activity. Overexpression of an uninhibitable GSK-3beta mutant resulted in partial inhibition of FGF-2-mediated cyclin D1 induction. The importance for cyclin D1 in FGF-2-induced angiogenesis in vivo is shown in cyclin D1-/- mice, where FGF-2-induced new vessel formation was significantly reduced compared with FGF-2-induced angiogenesis in cyclin D1+/+ mice. In conclusion, FGF-2 is a novel modulator of Lef/Tcf/beta-catenin signaling in endothelial cells, suggesting that angiogenic properties of FGF-2 are at least in part mediated by Lef/Tcf/beta-catenin activation.

0007

Vascular endothelial growth factor and psoriasis

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Psoriasis is associated with inappropriate vascular expansion in the upper dermis. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that promotes both angiogenesis and vascular permeability. VEGF is produced by epidermal keratinocytes and elevated levels are found in plaques of psoriasis. The VEGF gene is polymorphic and located at chromosome 6p.21, close to a known chromosomal locus for psoriasis susceptibility (*PSORS*1). The aim of this project was to investigate the frequencies of two VEGF polymorphisms at −460 (in the promoter region) and at +405 (in the 5'UTR), in patients with Type 1 (onset≦40 years) chronic plaque psoriasis compared to healthy controls and to measure plasma levels of VEGF and the soluble VEGF receptor (flt1). DNA was isolated from whole blood using Qiagen DNA kits (Qiagen Ltd UK) and VEGF genotyping performed by PCR–RFLP in 160 psoriatic patients and 102 healthy controls. Plasma VEGF and flt1 levels were assayed using validated ELISA kits (R&D Systems). There were no significant differences in genotype frequencies at the −460 or +405 sites comparing all psoriasis patients with controls. However, patients with severe disease (defined as PASI®12) showed significantly increased frequency of the +405 CC genotype (p•0.05) and C allele (p=0.028) compared to healthy controls. Patients with psoriasis onset between 20 and 40 years also showed significantly increased frequency of the +405 CC genotype (p•0.015) and C allele (p=0.016) compared to healthy controls. Plasma levels of VEGF and flt1 were significantly detectable in psoriatics compared with controls (p•0.001) and mean plasma levels of VEGF and flt1 were markedly elevated compared with controls. In summary, we have demonstrated a significant association of the VEGF +405 CC genotype and C allele with both severe psoriasis and age of disease onset. Plasma levels of VEGF and flt1 are increased in patients with psoriasis. These results may imply a pathogenic role for VEGF in psoriasis.

0008

UVB-induced skin angiogenesis is associated with a switch in the balance of vascular endothelial growth factor and thrombospondin-1 expression

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We have previously shown that targeted over-expression of the endogeneous angiogenesis inhibitor thrombospondin-1 (TSP-1) in the epidermis prevents chronic ultraviolet-B (UVB)-induced angiogenesis and cutaneous photo-damage in mice, suggesting that angiogenesis plays a critical role in the mediation of UVB effects on the skin. However, the molecular regulation of angiogenesis factors and inhibitors by acute UVB irradiation still remains to be elucidated. We performed quantitative analyses of cutaneous vascularity and of VEGF and TSP-1 expression after acute UVB irradiation of mouse skin. Skin vascularity in the upper dermis was greatly increased until day 8 after a single dose of UVB irradiation (200 mJ/cm2), associated with up-regulation of VEGF mRNA expression and with down-regulation of TSP-1 mRNA expression in the hyperplastic epidermis. After 13 days, skin vascularity was normalized with down-regulation of VEGF mRNA expression and up-regulation of TSP-1 mRNA expression to the levels in non-UVB irradiated skin. Double immunofluorescence stains revealed pronounced induction of the TSP-1 receptor CD36 in CD31-positive vessels at day 8 after UVB irradiation, associated with vascular endothelial cell apoptosis. These results demonstrate that acute UVB irradiation reverses the expression balance between VEGF and TSP-1, leading to shift towards a pro-angiogenic environment. They also suggest that both VEGF and TSP-1 play critical roles in the control of angiogenesis induced by acute UVB irradiation.

0009

Expression of the lymphatic-specific homeobox gene Prox1 in differentiated blood vascular endothelium induces the lymphatic endothelial cell phenotype

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It has been previously shown that polarized expression of the homeobox gene Prox1 in a subpopulation of venous endothelial cells of the embryonic cardinal vein promotes and maintains the budding of endothelial cells that will form the lymphatic vascular system. In Prox1 deficient mice, endothelial cells fail to acquire the lymphatic phenotype; instead, they remain as blood vascular endothelium. To investigate whether Prox1 is sufficient to induce a lymphatic fate in differentiated blood vascular endothelium, Prox1 cDNA was ectopically expressed by adenoviral gene transfer in primary human blood vascular endothelial cells and by transient plasmid cDNA transfection in immortalized microvascular endothelial cells. Transcriptional profiling combined with quantitative real-time RT-PCR and Western blotting analyses revealed that Prox1 expression upregulated the lymphatic endothelial cell markers podoplanin and vascular endothelial growth factor receptor-3. Conversely, genes such as laminin, VEGF-C, neuropilin-1 and intercellular adhesion molecule-1, whose expression has been associated with the blood vascular endothelial cell phenotype, were downregulated. These results were confirmed by the use of specific antibodies against some of these markers in sections of embryonic and adult tissues. These findings validate our previous proposal that Prox1 is a key player in the molecular pathway leading to the formation of lymphatic vasculature and identify Prox1 as a master switch in the program specifying lymphatic endothelial cell fate.

0010

TNFα induction of tissue factor in human dermal endothelial cells requires protein kinase CK2 activity

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Tissue factor (TF), or thromboplastin, is a cell surface receptor that serves as an initiator of blood coagulation and has been implicated in angiogenesis and tumor growth. The regulation of TF expression in endothelial cells is not fully defined and involves multiple signaling pathways. Previous studies have suggested that NF-kB activation, a critical event in TNFα-mediated TF induction, requires PI-3 kinase (P3K) activity. Other studies in large vessel endothelium have suggested that activation of P3K actually inhibits TF expression. In order to address the role of P3K in TF induction in skin microvascular endothelial cells, we examined the effect of LY294002 (LY) and wortmannin (WM), inhibitors of the P3K pathway, on TNFα-mediated TF induction. LY pretreatment partially blocked TNFα-mediated cell surface expression of TF in a concentration-dependent fashion. In contrast, WM pretreatment superinduced TF expression at the protein, steady-state mRNA, and transcriptional levels. Despite inhibiting TNFα-mediated increases in cell surface TF expression, LY had no effect on TNFα-mediated increases in TF mRNA or total cell TF protein as assessed by western blot. These data suggested that P3K activity was not essential for TNFα-mediated TF induction in HDMEC. Previous studies have suggested that LY is also an inhibitor of protein kinase CK2 (CK2), an activity that we confirmed using in vitro kinase assays with CK2-specific peptides. Since any LY effects on TF induction may be a consequence of inhibition of both P3K and CK2, we examined whether a specific inhibitor of CK2 would block TNFα-mediated TF induction. TBB, a specific ATP site-directed inhibitor of CK2, blocked TNFα-mediated induction of cell surface TF protein expression. This blockade was associated with inhibition of both total cell TF and TF mRNA induction. These data suggest that CK2 activity is critical for cytokine induction of TF and may provide a rationale for novel anti-TF therapy.

0011

Thrombospondin-2 deficiency accelerates UVB-induced angiogenesis and cutaneous photo-damage

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Chronic ultraviolet-B (UVB) irradiation of the skin results in epidermal hyperplasia, degradation of extracellular matrix molecules, formation of wrinkles and pronounced angiogenesis. We have previously reported that the cutaneous vascular system plays a critical role in the mediation of UVB induced skin damage, and that over-expression of thrombospondin-1 in the epidermis prevents chronic skin damage and angiogenesis. Here, we studied the biological function of the related endogeneous angiogenesis inhibitor thrombospondin-2 (TSP-2) in the pathology of UVB induced skin damage. We subjected TSP-2 deficient mice and wild-type control mice to a twelve weeks UVB irradiation regimen. After twelve weeks UVB irradiation (cumulative UVB dose: 7.23 J/cm2), TSP-2 deficient mice showed pronounced wrinkle formation and increased angiogenesis with disorganized collagen fibers and increased inflammatory cell infiltration in the dermis, as compared with wild-type controls. Immunohistochemistry for CD31 and computer-assisted image analysis of CD31 stained sections revealed increased skin vascularization and proliferation of endothelial cells in TSP-2 deficient mice after chronic UVB irradiation. Moreover, in situ hybridization revealed a strong up-regulation of VEGF mRNA expression in the suprabasal keratinocytes of the epidermis of TSP-2 deficient mice after chronic UVB irradiation as compared with wild-type mice. These results demonstrate that lack of TSP-2 results in enhanced angiogenesis, leading to increased skin damage after chronic UVB irradiation, They also suggest that TSP-2 plays a protective role in the control of cutaneous photo-damage.

0012

P-selectin expression in human dermal microvascular endothelial cells

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P-selectin, a member of the selectin family of adhesion receptors, is a 140-kDa glycoprotein stored in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. Upon cellular activation, P-selectin is rapidly redistributed from secretory membranes to the surface of endothelial cells, where it mediates rolling of neutrophils, lymphocytes and eosinophils. Whereas the regulation of P-selectin expression has been intensively studied on cultured human umbilical vein endothelial cells (HUVEC), the kinetics and mechanism of P-selectin expression in human dermal microvascular endothelial cells (HDMEC) are still unclear. In this study, we analyzed the regulatory mechanisms of P-selectin expression on cultured HDMEC by flowcytometry and cell-based ELISA. Histamine stimulated HDMEC to express P-selectin within minutes. IL-4, IL-13 and substance P induced a marked increase in P-selectin expression after 24 hr in culture. In contrast, neither TNFα, IL-1α nor INF-γexhibited any effect on P-selectin expression in HDMEC as assessed by cell-based ELISA. Laser scanning microscopy examination revealed constitutive P-selectin expression on the luminal surface of dermal endothelial cells in normal skin. The increased luminal exopression of P-selecitin was observed in skin lesion of urticaria, atopic dermatitis and bullous pemphigoid but not psoriasis vulgaris and P.P.P. These data indicate that constitutive luminal expression of P-selectin in dermal endothelial cells may be involved in the leukocyte trafficking. IL-4, IL-13, substance P as well as histamine could promote the interaction of leukocytes with dermal endothelial cells via enhancing P-selectin expression.

0013

Pharmacologic evidence of an autocrine angiopoietin2-tie-2 loop in the pathogenesis of hemangioma of infancy

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Hemangiomas of infancy represent the most common tumor of childhood. Treatment of large hemangiomas remains unsatisfactory, and may lead to significant morbidity. We hypothesized that autocrine loops may play a role in hemangioma formation. We found that the endothelial specific receptor, tie-2, is constitutively activated in human hemangiomas. Using a murine model of hemangioma, bend3 cells, we found that hemangiomas in mice express both tie-2, and its ligand, angiopoietin-2. In order to determine whether tie-2/angiopoietin-2 interactions are functional in vivo, we infected bend3 cells with a recombinant adenovirus expressing a soluble tie-2/Fc protein, or as a control, adenovirus expressing Fc protein alone. Expression of soluble tie-2/Fc led to a significant inhibition of hemangioma formation in mice, compared with Fc fragment alone. Our findings suggest that tie-2/angiopoietin loops are functionally important in the development of human hemangiomas, and that pharmacologic inhibition of these autocrine loops may lead to improved treatment for hemangiomas

0014

The endogenous angiogenesis inhibitor thrombospondin 1 is up-regulated in acute allergic contact dermatitis in human skin

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In cutaneous inflammatory diseases such as eczema and psoriasis, increased vascularity and vascular hyperpermeability are characteristic features. Vascular endothelial growth factor A (VEGF-A) and other proangiogenic growth factors are potently upregulated in inflamed lesions, conversely, the endogenous angiogenesis inhibitor thrombospondin 2 (TSP)-2 was recently found to play an important role in the control of an experimental delayed-type hypersensitivity reaction in the skin of mice. In order to investigate the biologic role of angiogenesis in human cutaneous inflammation, we quantified the vascularity (number, size and density) in acute allergic contact dermatitis and normal skin using computer-assisted morphometric image analysis of collagen-IV and CD31(PECAM-1)-stained frozen sections. Furthermore, we compared the levels of TSP-1 expression in inflamed lesions and healthy skin from the same donor. We report that vessel density in the inflamed skin was already increased 72 hours after the onset of an inflammation (our earliest timepoint). In the inflamed lesions, we found TSP-1 protein expression in the basement membrane, in some extravasated leukocytes but mainly in blood vessels surrounded by perivascular infiltrate. TSP-1 mRNA at this timepoint was confined to these blood vessels. In normal skin, TSP-1 expression was found in the basement membrane and in some basal keratinocytes of the hair follicle. CD36, one of the receptors for TSP-1, was also upregulated in the inflamed lesions and was expressed primarily by inflammatory cells. Our data implicate TSP-1 as a modulator of endothelial cell function during an inflammation. Further characterization of additional receptors, as well as investigating the time course of TSP-1 upregulation in these lesions will elucidate the role of TSP-1 during an inflammatory response.

0015

Identification of vascular lineage-specific genes by transcriptional profiling of isolated cutaneous blood vascular and lymphatic endothelial cells

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In mammals, the lymphatic vascular system develops by budding of lymphatic progenitor endothelial cells from embryonic veins to form a distinct network of draining vessels with important functions in the immune response and in cancer metastasis. However, the lineage-specific molecular characteristics of blood vascular versus lymphatic endothelium have remained poorly defined. Comparative gene array analyses of cultured BVEC and LEC revealed that only 1.6% of genes showed an at least 2-fold increased expression in LEC (1.2% in BVEC), including Prox1 and podoplanin as known lymphatic markers, in agreement with the close lineage connection between both types of vascular cells. Transcriptional profiling studies revealed increased expression of several extracellular matrix and adhesion molecules in BVEC, including versican, collagens, laminin and N-cadherin, and of the growth factor receptors endoglin and vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1). Quantitative real-time RT-PCR and differential immunostains of human skin confirmed the blood vessel-specific expression of these genes. During embryonic development, endoglin expression was gradually downregulated on lymphatic endothelium whereas VEGFR-1 was absent from lymphatics. We also identified several genes with specific expression in LEC. These results demonstrate that some lineage-specific genes are only expressed during distinct developmental stages and they identify new molecular markers for blood vascular and lymphatic endothelium with important implications for future studies of vascular development and function.

0016

Constitutive transgenic expression of dominant negative tuberin leads to a skin and brain phenotype in mice

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Tuberous sclerosis (TS) is a common autosomal disorder caused by loss or malfunction of tuberin or hamartin. Many tumors in TS do not demonstrate loss of heterozygosity (LOH) for these genes, implying that dominant negative forms of these genes may account for some neoplasms in TS. In order to test this hypothesis, we expressed a dominant negative allele of tuberin (ΔRG) behind a CMV promoter in NIH3T3 cells and in transgenic mice. This allele binds hamartin, but has a deletion in the C-terminal protein, leading to constitutive activation of rap1 and rab5/rabaptin. Expression of ΔRG in 3T3 cells led to a strong induction of reactive oxygen species (ROS), induction of VEGF, and malignant transformation in vivo. Expression of ΔRG in transgenic mice led to high level expression in all murine tissues examined, including skin, kidney, liver, and brain. Surprisingly, mice developed a fibrovascular collagenoma, which closely resembles Shagreens patches observed in human patients with TS. In addition, deficient migration of periventricular external granule cells in the brain was observed, which may be precursors of the subependymal giant cell astrocytomas seen in TS patients. Thus, expression of a dominant negative tuberin in all tissues can lead to a tissue-specific phenotype resembling some of the findings in human TS. Our data is the first to suggest that specific signaling abnormalities may underlie specific pathology (Shagreens patch subependymal giant cell astrocytoma) in a human genetic disorder

0017

Treatment with the angiogenesis inhibitor vasostatin decreases vascularity and edema formation in an experimental delayed-type hypersensitivity reaction in the skin of mice

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Angiogenesis, increased vascular hyperpermeability and upregulation of proangiogenic factors are characteristic features of several inflammatory cutaneous diseases. Recently, mice that overexpress the proangiogenic agent placental growth factor (PlGF) were reported to have increased cutaneous inflammation. Conversely, PlGF knockout mice demonstrated a diminished inflammatory response while mice that lack the endogenous angiogenesis inhibitor thrombospondin 2 exhibited a persistent and prolonged inflammation. Yet, little is known about the effect of antiangiogenic treatment on the timecourse and extent of a cutaneous inflammation. Vasostatin is the 180 amino acid NH2 domain of calreticulin known to inhibit angiogenesis in vitro and in vivo and has been shown to potently suppress tumor growth. We induced a delayed-type hypersensitivity (DTH) reaction in the ear skin of wildtype FVB mice with and without vasostatin treatment in order to investigate any potential alleviative effect resulting from the suppression of angiogenesis. We report that 24 and 48 hours after the initial challenge, the vasostatin-treated mice exhibited 38% and 31% less edema respectively when compared to the untreated mice. This trend was observed throughout the investigated recovery period of 7 days where the treated mice demonstrated 50% less inflammation starting 72 hours post challenge. Using computer-assisted morphometric image analysis of CD31(PECAM-1)-stained frozen sections, we found decreased vascularity in the vasostatin-treated mice 24 and 48 hours after challenge. Our results reveal an important role for angiogenesis during an inflammatory response and suggest that therapeutic approaches aimed at maintaining vascular quiescence could prove beneficial in the treatment and prevention of cutaneous inflammations.

0018

Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival

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Malignant melanomas of the skin are distinguished by their propensity for early metastatic spread via lymphatic vessels to regional lymph nodes, and lymph node metastasis is a major determinant for the staging and clinical management of melanoma. However, the importance of tumor-induced lymphangiogenesis for lymphatic melanoma spread has remained unclear. We investigated whether tumor lymphangiogenesis occurs in human malignant melanomas of the skin and whether the extent of tumor lymphangiogenesis may be related to the risk for lymph node metastasis and to patient survival, using double immunostains for the novel lymphatic endothelial marker LYVE-1 and for the panvascular marker CD31. Tumor samples were obtained from clinically and histologically closely matched cases of primary melanomas with early lymph node metastasis (n=18) and from non-metastatic melanomas (n=19). Hot spots of proliferating intratumoral and peritumoral lymphatic vessels were detected in a large number of melanomas. The incidence of intratumoral lymphatics was significantly higher in metastatic melanomas and correlated with poor disease-free survival. Metastatic melanomas had significantly more and larger tumor-associated lymphatic vessels, and a relative lymphatic vessel area of greater than 1.5% was significantly associated with poor disease-free and overall survival. In contrast, no differences in the density of tumor-associated blood vessels were found. VEGF and VEGF-C expression was equally detected in a minority of cases in both groups. Our results reveal tumor lymphangiogenesis as a novel prognostic indicator for the risk of lymph node metastasis in cutaneous melanoma.

0019

An extract of the root of *Lithospermun erythrorhison* accelerates wound healing in diabetic mice

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Many people suffer from intractable bedsores, which sometimes develop because of chronic metabolic failure in patients. An extract of the root of *Lithospermun erythrorhison* (SK) has been reported to have an effect on wound healing. However, the effects of SK have not been studied in chronic wounds, such as bedsores. The healing-impaired diabetic (*db/db*) mouse is a good model for the investigation of clinical healing therapies. Therefore, we examined whether SK accelerates wound healing in db/db mice. Full-thickness round wounds of 6-mm diameter were created on the backs of mice. After applying SK, we covered the wound with a film dressing to keep it moist. At three weeks, wound closure was complete in SK-treated mice but not in controls. Capillary vessel number and collagen synthesis increased early in wound healing in SK-treated wounds. At this time, vascular endothelial growth factor (VEGF)-positive neutrophils had infiltrated the wound and the appearance of apoptotic fibroblasts and endothelial cells in the granulation tissue was more advanced than in the controls. Where the wound was covered with epithelium, infiltration of VEGF-positive cells and apoptotic cells tended to decrease. These results suggest that the inflammatory phase was shortened, and the proliferative and maturation phases were advanced by SK. It has been known that SK also has anti-bacterial activity. Therefore, we conclude that SK is useful for wound healing in *db/db* mice, and could potentially help patients with intractable bedsores.

0020

Mice overexpressing placenta growth factor and lacking vascular endothelial growth factor in the skin exhibit increased vascularization but normal vessel permeability

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Among the members of the vascular endothelial growth factor family (VEGF), placental growth factor (PlGF) has recently been demonstrated to have strong angiogenic and permeabilizing properties in animal models. However, it has not been clarified, yet, whether PlGF exerts these effects only by enhancing VEGF-driven angiogenesis and permeability or also by activating its own receptor. To address this issue in an *in vivo* model, mice overexpressing PlGF and lacking VEGF in the skin (PlGF+/VEGF-) were obtained by crossing mice overexpressing murine PlGF under the control of the keratin 14 promoter (PlGF+) and mice with keratin 5-Cre/LoxP-mediated deletion of VEGF (VEGF-). Skin vascularization was investigated by analyzing whole mount ear preparations after perfusion with *Lycopersicon esculentum* lectin and by immunohistochemical staining with PECAM-1 and alpha-smooth muscle actin of skin sections from the back. PlGF+/VEGF- as well as PlGF+ mice had an increased number of vessels ($p<0.01$), with augmented diameter ($p<0.05$) and branching ($p<0.01$) in comparison with wild type littermates, and showed features of active angiogenesis, such as glomeruloid bodies and spikes. In agreement with these data, the area of PECAM-1 positive vessels in back skin sections was increased in PlGF /VEGF- and PlGF+ mice in comparison with wild type mice ($p<0.05$). Vessel permeability was studied by evaluating leakage sites in *Ricinus communis* perfused whole mount ear preparations, and by transmission electron microscopy of back skin. In comparison with wild type mice, the number of permeability sites was not modified in PlGF+/VEGF- animals, while it was markedly increased in PlGF+ mice ($p<0.01$). Our data indicate that, in the absence of keratinocyte-derived VEGF, PlGF overexpression can promote skin angiogenesis, while it is not able to increase vessel permeability.

0021

Paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a humanized SCID mouse model

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Paclitaxel is an alkaloid which inhibits endothelial cell proliferation, motility and tube formation at nanomolar concentrations. Cationic liposome preparations have been shown to target blood vessels. We wished to explore the possibility that paclitaxel encapsulated in cationic liposomes carries paclitaxel to blood vessels and thereby provides an anti-angiogenic effect. We used a humanized SCID mouse melanoma model, which allows to analyze tumor growth and tumor angiogenesis in an orthotopic tumor model. Here, human melanoma cells grow on human dermis and are in part nourished by human vessels. We show that paclitaxel encapsulated in liposomes prevents melanoma growth and invasiveness and improves survival of mice. Moreover, liposome-encapsulated paclitaxel reduces vessel density at the interface between the tumor and the human dermis and reduces endothelial cell mitosis to background levels. In contrast, equimolar concentrations of paclitaxel solubilized in Cremophor EL had only insignificant effects on tumor growth and did not reduce the mitotic index of endothelium in vivo, although the anti-proliferative effect of solubilized paclitaxel in Cremophor EL in vitro was identical to that seen with liposome-coupled paclitaxel. In conclusion, we present a model of how to exploit cytotoxic effects of compounds to prevent tumor growth by using cationic liposomes for targeting an anti-proliferative drug to blood vessels.

0022

Sodium lauryl sulfate induces VEGF expression in immortalized keratinocytes via an egr-1, MEK1-dependent mechanism

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Irritant contact dermatitis (ICD) is characterized acutely by erythema and edema evolving with chronic exposure to hyperkeratosis and lichenification. The cellular and molecular mechanisms involved in ICD are poorly understood. Keratinocytes are a major target for irritants resulting in activation and secretion of soluble mediators e.g vascular endothelial growth factor (VEGF), a powerful pro-inflammatory and angiogenic agent. We determined whether sodium lauryl sulfate (SLS), a model irritant, induces VEGF expression in HaCat cells at non-toxic concentrations. In order to best define the appropriate dosing for maximum cell viability, testing with SLS was performed at different concentrations (0.1-0.001%) and exposure times (5-60 minutes). Five minute exposure to concentrations of SLS as high as 0.01% resulted in minimal effects on cell viability. Under these conditions, VEGF mRNA expression was upregulated 5-30 fold after one hour, responses that were comparable to TGFα treatment. Using oligonucleotide probes from the egr-1 responsive region of the VEGF promoter in EMSA, we demonstrated that SLS treatment resulted in the appearance of nuclear complexes that bound the VEGF promoter derived EMSA probe. Supershift analysis demonstrated these complexes contained egr-1 protein. SLS treatment resulted in increased levels of steady state egr-1 mRNA, suggesting that SLS may target egr-1 gene expression. As previous studies have linked egr-1 induction to MEK1 activation, we examined whether PD98059, a specific inhibitor of MEK1, blocked SLS-induced VEGF expression. Pretreatment of HaCat cells with PD98059 prior to SLS treatment blocked VEGF induction as well as SLS mediated egr-1 expression, whereas the proteasomal inhibitor MG-132 had no effect. These data suggest that irritants such as SLS may target MEK1 activation and egr-1 induction. These studies provide insight into novel approaches for the development of relevant in vitro test systems to investigate the mechanisms and prevention of ICD.

0023

Transgenic VEGF overexpression induces enlargement of cutaneous lymphatic vessels that is further enhanced by delayed-type hypersensitivity reactions

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Lymphatic vessels (LV) are pivotal for the maintenance of fluid balance in the skin. Under physiologic conditions, most LV are collapsed, whereas in inflammation, LV are dilated in response to the increased vascular leakage and edema formation. Vascular endothelial growth factor-A (VEGF-A) has been shown to be upregulated in inflammatory skin diseases including psoriasis. Whereas the importance of VEGF-A as an inducer of blood vessel permeability and angiogenesis is well established, its influence on LV has remained unknown. We investigated delayed-type hypersensitivity reactions (DTH) in the ear skin of transgenic mice with epidermis-specific overexpression of VEGF-A and in wild-type littermates. Immunostains for the lymphatic-specific hyaluronan receptor LYVE-1 revealed enlargement of dermal LV in VEGF-A transgenic mice already in non-inflamed skin. These findings were confirmed by in vivo fluorescence confocal microscopy. Elicitation of DTH reactions led to a significantly more pronounced edema formation and LV enlargement in VEGF-A transgenic mice as compared with wild-type controls. Whereas tissue swelling and dilation of LV disappeared within 7 days after elicitation in wild-type mice, they persisted for more than 2 weeks in VEGF-A overexpressing mice. Double labeling for LYVE-1 and for the lymphatic-specific homeobox gene Prox-1 confirmed the lymphatic identity of the dilated vessels. After challenge, VEGF-A transgenic mice developed chronic inflammatory skin lesions resembling psoriasis. This effect persisted even after vascular permeability had largely returned to baseline levels. Our findings reveal that VEGF-A, in addition to its known effects on blood vessel angiogenesis, also exerts potent effects on cutaneous lymphatic vessels.

0024

Podoplanin/T1-alpha deficiency disrupts lymphatic patterning and causes lymphedema

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Within the vascular system, the mucin-type transmembrane glycoprotein podoplanin/T1alpha is predominantly expressed by lymphatic vascular endothelium, and recent studies have shown that it is regulated by the lymphatic-specific homeobox gene Prox1. We examined the role of podoplanin in vascular development by studying its expression pattern and the effects of gene disruption in mice. Within the vascular system, podoplanin is first expressed around E11.0 and in a subset of budding Prox1-positive lymphatic progenitor cells. Expression is predominantly restricted to the lymphatic endothelium during later development, and ultrastructural analysis revealed its localization to the luminal and abluminal plasma membrane of lymphatic vessels. Podoplanin-null mice have defects in lymphatic, but not blood vessel pattern formation. These defects are associated with diminished lymphatic transport, congenital lymphedema and dilation of cutaneous and intestinal lymphatic vessels. Podoplanin was also weakly expressed in the basal epidermis of newborn wild-type mice, but gene disruption did not alter epidermal differentiation. Studies in cultured endothelial cells revealed that podoplanin promotes cell adhesion and migration. This glycoprotein therefore appears to be important for endothelial cell migration and patterning of the lymphatic vascular system.

0025

Identification of proteins expressed in oxidative stress-induced states in cultured human dermal microvascular endothelial cells

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Aerobic metabolism has been known to produce reactive oxygen species (ROS) that are regarded as toxic byproducts. However, ROS can also act as intracellular signaling molecules in vascular cells and they can mediate phenotype changes in vascular endothelial cells that may be considered both physiological and pathological. To clarify the molecular mechanisms of ROS signaling, we examined the H2O2-responsive proteins using proteomics tools. Changes in the protein expression were studied in cultured human dermal microvascular endothelial cells exposed to low- and high-level hydrogen peroxide for various time intervals and intracellular ROS production was examined using flow cytometry and spectrophotometer. Proteins were separated from dose dependent and time course series experimental conditions by two-dimensional electrophoresis, and they were visualized by silver staining and quantified by image processing. The proteins of interest were subjected to in-gel digestion with trypsin, and the masses of resulting peptides were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This tryptic mass map was used to identify the proteins through the search of a protein-sequence database. At least 117 proteins were stimulated by H2O2, whereas 46 proteins were repressed by this treatment. The majority of changes occurred within the first 15 min of cell stimulation. Oxidative stress challenge results in a dramatic change of protein profile and their mapping may give a global view of the cellular changes elicited by H2O2. The results could provide the framework for understanding the mechanisms of cellular redox homeostasis and H2O2 metabolism.

0026

Signaling events during induction of PAI-1 expression by SPC in cultured human dermal fibroblasts

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Sphingosylphosphorylcholine (SPC) is a bioactive lipid stimulating wound healing. Although SPC is known to act as a mitogen in wound healing, the exact mechanism how to accelerate wound healing is not fully elucidated. Plasminogen activator inhibiter-1 (PAI-1) is also well known to affect wound healing by regulating the fibrinolytic environment of the wound area. Under the hypothesis that SPC could affect wound healing through PAI-1, we tested PAI-1 expression by SPC and found that SPC stimulated the expression of PAI-1 in human dermal fibroblasts at the protein and mRNA levels. SPC induced an oscillatory increase in the intracellular calcium concentration ([Ca2+]) in human dermal fibroblasts. Preincubation with thapsigargin, but not with EGTA ameliorated PAI-1 induction by SPC, suggesting that an increase of intracellular [Ca2+] from internal stores could mediate PAI-1 induction by SPC. This effect of SPC on PAI-1 expression through increasing intracellular [Ca2+] could be inhibited by the blocking of Gi-coupled-protein receptor prior to treatment of SPC, but not by blocking 00 the tyrosine kinase receptor. Although PKC activation by phorbol-12-myristate-13-acetate alone could induce PAI-1 production, deactivation of PKC could not block induction of PAI-1 production by SPC. SPC also induced MAPK phosphorylation of p42/44 ERK in human dermal fibroblasts and pretreatment with PD98059 for blocking of the MEK pathway could attenuate SPC-induced PAI-1 expression. Therefore, we could reach a conclusion that SPC could affect the wound healing through PAI-1 induction mediated by a Gi-protein-coupled receptor and ERK, with an increase in the intracellular Ca2+ level.

0027

Determination of the functional integrity of skin autonomous vascular regulation with laser Doppler technique

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Local and systemic regulatory mechanisms are responsible for maintaining proper skin blood supply. Failure of the skin autonomous vascular regulation poses severe problems in diseases like lymphoedema, diabetes mellitus, leg ulcers, Raynaud syndrome, etc. Determination of the integrity of cutaneous autonomous regulation of blood supply could be an important prognostic factor in the treatment of these diseases. We aimed to develop a non-invasive method for monitoring the integrity of skin autonomous vascular regulation. First we used baroreflexes under physiological conditions to initiate changes in skin blood flow. After this we used bretylium locally to neutralize sympathetic activity by presynaptically blocking noradrenalin release. Servo-photopletysmography was used to monitor haemodynamic changes, while laser Doppler technique was used for measurement of skin blood flow. Without bretylium treatment cutaneous vascular resistance showed a significant, threefold increase in the hypotonic phase of the baroreflex maneuver. Bretylium treatment completely inhibited this increase. Our data indicate that baroreflex stimulus results in vasoconstriction of skin arterioles and this vasoconstriction is the result of sympathetic efferent activity. We propose that monitoring of cutaneous vascular resistance over bretylium treated and non-treated skin areas is a convenient and non-invasive method to determine the functional integrity of skin autonomous regulation of blood supply.

0028

Keratin5-Cre/LoxP-mediated inactivation of vascular endothelial growth factor sensitizes mouse skin to UVB-induced photodamage

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Excessive exposure of skin to sunlight results in erythema, dilation of dermal blood vessels and vascular hyperpermeability, suggesting that these changes in the dermal vasculature are necessary components of the protective response to UV-induced photodamage. Vascular Endothelial Growth Factor (VEGF) is one of several pro-angiogenic factors that are induced in skin after UVB irradiation, and epidermal keratinocytes (KC) are the major source of VEGF in skin. Using the Cre/LoxP system under the control of the keratin5 promoter, we have generated mice in which VEGF has been inactivated in epidermal KC (VEGF-AΔk5-cre/Δk5-cre), and used these animals to study the contribution of KC-derived VEGF to acute UVB-induced photodamage. We found that these mice developed burn-like lesions after a single UVB irradiation, at a dose at which the control mice were unaffected. At higher doses, at which the control mice also showed acute photo-damage, the VEGF-AΔk5-cre/Δk5-cre mice developed the lesions earlier and healed more slowly. Microscopic examination of the irradiated skin revealed massive inflammation, with complete loss of the epidermis in the mutant but not in the control mice, and CD31 stained blood vessels were missing in the vicinity of the basal membrane of mutant mice. Active caspase 3 staining revealed more apoptotic cells in the dermis, but lower numbers in the epidermis of mutant mice, suggesting that the latter may have died by necrosis. Proliferating cells were reduced in the epidermis of the mutant mice. Short-term repeated irradiation with a lower dose of UVB did not induce open wounds in old mice, but resulted in a scar-like lesion only in mutant mice. In addition they showed more pronounced hyperplasia, lack of sub-epidermal blood vessels and increased number of apoptotic cells in the epidermis. These results suggest that in the absence VEGF in epidermal KC, skin is sensitized to UVB-induced photodamage.

0029

Enhanced expression of HIF1alpha in squamous cell carcinoma

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The purpose of this study is to better define the role of hypoxia inducible factor 1-alpha (HIF1alpha) in epidermal tumorigenesis. HIF1alpha is a transcription factor that regulates cellular adaptive responses to hypoxia including the expression of angiogenic factors such as vascular endothelial growth factor (VEGF). In the current study, we have investigated the regulation of HIF1alpha mRNA expression in human keratinocytes by hypoxic conditions in vitro and we have evaluated the levels of HIF1alpha and VEGF protein in squamous cell carcinomas in vivo. In the first set of experiments, pre-confluent primary human keratinocyte cultures derived from adult surgical specimens were exposed to hypoxia (<2% O_2) or normoxia (21% O_2) for 48 hours and then harvested for total RNA. Northern analysis demonstrated a significant increase in the levels of HIF1alpha-specific mRNA in the hypoxic cells; this was confirmed by quantitative RT-PCR to represent a 6.1±1.9-fold increase over normoxic controls. In the subsequent set of experiments, histological sections obtained from squamous cell carcinoma biopsy specimens underwent immunohistochemical analysis using antibodies directed against HIF1alpha and VEGF. Immunostaining patterns demonstrated markedly enhanced HIF1alpha protein levels in the acanthotic tumor epithelium when compared to adjacent uninvolved epithelium. Staining was particularly enhanced in areas surrounding vascular channels within the tumor mass and around areas of necrosis. Immunostaining for VEGF demonstrated an identical pattern. Thus, it appears likely that hypoxia encountered in an expanding squamous cell carcinoma results in enhanced HIF1alpha expression which drives VEGF expression stimulating the angiogenic response necessary for sustaining tumor growth.

0030

Treatment of facial angiofibromas with topical pimecrolimus and tazarotene gel

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New insights into the pathogenesis of tuberous sclerosis-associated skin lesions suggest that angiofibromas are angiogenic neoplasms. Our purpose was to treat facial angiofibromas in patients with tuberous sclerosis with topical agents known to have antiangiogenic activity. Three patients were treated with a regimen of pimecrolimus every morning and tazarotene 0.1% gel every evening. At two months, patients were evaluated clinically. To date, two patients have been evaluated after two months of treatment and in both there has been a significant reduction in the size and color of the angiofibromas. Historically, topical treatment of facial angiofibromas has been disappointing. Current treatment of angiofibromas with ablative and vascular lasers, while effective at treating existing lesions, has not been shown to prevent the development of new lesions. Ideal treatment of angiofibromas would not only treat existing lesions but also prevent the development of new angiofibromas. Recently, studies on a rat model with kidney tumors associated with tuberous sclerosis have shown that rapamycin has an effect on the tumors with induction of apoptosis and a reduction in cell proliferation. Rapamycin like pimecrolimus and tacrolimus are macrolide antibiotics which bind FKBP (FK506 binding protein) and inhibit the calcineurin pathway. Pimecrolimus is a topical immunomodulator approved for use in atopic dermatitis. Lastly, retinoids were chosen because they have been shown to have antiangiogenic activity. We offer a new approach to the topical treatment of angiofibromas with the combined use of pimecrolimus cream and tazarotene gel. We acknowledge the small number of patients in our study, and will continue to expand on the series.

0031

Formation of microvessels in tissue equivalents using blood-derived endothelial precursor cells

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We previously demonstrated that human skin equivalents can be vascularized and perfused through microvessels lined by cultured human endothelial cells (EC). Such cultured EC are generally allogeneic (or xenogeneic) to the graft recipient and may induce a destructive immune response. We have now identified conditions for forming perfused microvessels using EC derived from blood precursor cells. Endothelial precursor cells (EPC) were propagated from human umbilical cord blood by incubation in selective culture media. In the first 10-14 days, the cells elongated, but did not divide. Thereafter, they developed a uniform spindle-shaped morphology and proliferated, displaying a growth pattern typical of EC. After passage, 95% were confirmed to be EC by flow cytometric detection of CD31 and TNF induced E-selectin expression. These EC cultured from umbilical cord blood formed capillary-like tubes when suspended in 3D culture in collagen/fibronectin gel and also migrated into devitalized human dermis in vitro, displaying behavior equivalent to cultured EC isolated from blood vessels. Differentiated EPC seeded in collagen/fibronectin gel or onto the underside of acellular dermis were implanted subcutaneously into immunodeficient mice. One month after engraftment, 3 of 6 gel and 3 of 3 dermal tissue-like implants were observed to contain multiple microvessels. These vessels were reactive with anti-human CD31 antibody and contained erythrocytes, indicating that they were both lined by human EC and had inosculated with the mouse circulation. Perfused microvessels were also successfully formed in vivo in 5 of 5 subcutaneously implanted collagen/fibronectin gels seeded with EPC transduced with Bcl-2, an anti-apoptotic gene, which we have shown to augment vascular maturation in tissue equivalents. Forming microvessels from blood derived EPC in vivo offers an approach to vascularize synthetic tissue, such as skin equivalents, with a source of EC autologous to the recipient.

0032

UV down-regulates the angiogenesis inhibitor thrombospondin-1 in normal human keratinocytes

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Exposure of human skin to ultraviolet (UV) irradiation results in cutaneous angiogenesis. A balance between positive and negative angiogenic factors maintains vascular homeostasis and when disrupted, angiogenesis ensues. The proangiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and interleukin-8 are upregulated within hours of physiologic UVB irradiation to skin. However, the effect of UVB on angiogenic inhibitors thrombospondin (TSP)-1 and -2 is not known. To determine UV irradiation effect on TSP-1 and 2, normal human keratinocytes were exposed to increasing doses of solar simulated irradiation (5 -50mJ/cm^2, metered at 285±5nm) or were sham irradiated. Total cellular RNA, proteins and cellular supernatants were collected at different intervals after irradiation and processed for northern and western blot analyses. There were no consistent modulations of TSP-1 expression in sham irradtiated cells or in cells irradiated with 5 and 15mJ/cm^2. However, within 1hr, TSP-1 mRNA was downregulated with 30 and 50mJ/cm^2, respectively. Maximal downregulation of 75 % was observed within 6 through 24 hrs with 50 mJ/cm^2. TSP-1 returned to baseline levels within 48 hrs after irradiation. However interestingly, within 72 and through 96 hrs, TSP-1 expression was upregulated with 30 and 50 mJ/cm^2. Western blot analysis of cell lysates for TSP-2 did not show consistent modulations. However, because TSP-2 is primarily a secreted protein, TSP-2 levels were determined in cell supernatants. Within 96 hrs there was a substantial decrease of TSP-2 levels in UV irradiated supernatants. Our findings confirm that both TSP-1 and 2, the major angiogenic inhibitors in skin, are expressed and synthesized by normal human keratinocytes. Furthermore, UV irradiation downregulates TSP-1 mRNA and decreases TSP-2 secretion. In addition to angiogenic factors, TSP-1 and 2 may have an important role in the pathophysiology of UV-induced angiogenesis in a variety of cutaneous disorders.

0033

Lymphatic microvascular endothelial cells are specialized in scavenging and killing microbial pathogens: a protective role for the lymph node environment?

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Lymphatic microvascular endothelial cells (LECs) are interposed between cutaneous tissues and the lymph node (LN) environment. A well-established mode of antigen transport from the skin to the LN is the shuttling of dendritic cell (DC)-bound antigen via dermal lymphatic vessels. In this study, we sought to investigate whether and how soluble, non DC-bound pathogens are handled by LECs. Capitalizing on the recent advancement of culturing purified dermal LECs, gene chip based profiling of podoplanin+/VE-cadherin+/CD31+/CD45- LECs revealed the expression of a large repertoire of antigen uptake receptors, among them the macrophage mannose receptor, endosialin and the c-type lectin P1. To investigate the functional capacity of LECs to bind, take up, and clear microbial pathogens, LECs were exposed to FITC-labeled yeast, E. coli and S. aureus bioparticles. LECs efficiently bound those microbes and internalized them with an average surface half-life of less than 60 min as demonstrated by trypan blue quenching of extracellular FITC moieties. Once internalized, pathogens or their degradation products remained cell-associated since 90% of the ingested bioparticle-bound I125 activity was retained in LECs for at least 48h. Importantly, we demonstrated in classical killing experiments that LECs are capable of eliminating E. coli. The killing efficacy is, however, less pronounced than that of macrophages or monocyte-derived DCs. Taken together, we demonstrate that LECs express a plethora of antigen uptake receptors and in line with this finding, are capable of binding, ingesting, and killing microbial pathogens. We speculate that LECs build up a functional pattern recognition receptor decorated barrier specialized to shield lymph nodes from damage through invading microorganisms.

0034

Rapamycin inhibits growth of human malignant melanoma in an animal xenograft model

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We examined effectiveness of rapamycin as an anti-melanoma agent in NOD scid mice bearing human tumors. Rapamycin is a novel immunosuppressive agent that inhibits signaling through the mammalian Target Of Rapamycin (mTOR). Recently, rapamycin has received renewed attention as a potential anti-tumor agent following reports demonstrating a role for mTOR in regulating expression of Vascular Endothelial Growth Factor (VEGF), one of the most potent angiogenic factors. We injected NOD scid mice subcutaneously with human A375 melanoma cell line. 100% of injected mice developed tumors. As tumors approached measurable volume (2 mm diameter), mock treatment or treatment with rapamycin by daily intraperitoneal injections (2 mg/kg/day) was initiated. Whereas tumors in untreated mice displayed typical logarithmic growth, tumors in rapamycin treated animals initially exhibited slow growth followed by growth arrest. At the time of sacrifice the average tumor volume in untreated animals was 4000 mm^3, whereas in rapamycin treated animals tumor volumes reached a plateau of 500 mm^3. Interestingly, rapamycin discontinuation resulted in renewed tumor growth. However, growth could again be arrested by rapamycin re-treatment. This response strongly suggests an anti-angiogenic mechanism of action. Indeed, A375 cells in culture produced significant levels of VEGF, which was strongly inhibited by rapamycin treatment. The effects of tumor-produced VEGF are likely limited to the tumor microenvironment as no significant increase in VEGF in the serum of tumor-bearing mice was observed. Immunohistochemical analysis of tumors demonstrated significant decrease in the number and size of intratumoral blood vessels in response to rapamycin, consistent with inhibition of tumor angiogenesis. These studies provide further evidence for effectiveness of anti-angiogenic agents such as rapamycin for treatment of melanoma.

0035

***In vivo* model of adhesion molecule regulation in synthetic human microvessels**

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Human umbilical vein endothelial cells (HUVEC) that have been retrovirally transduced to overexpress Bcl-2, suspended in collagen-fibronectin gels, and implanted into CB.17 SCID/beige mice form mouse smooth muscle/pericyte-invested, human EC-lined microvessels containing elements which morphologically resemble arterioles, capillaries, and venules. In normal human skin, TNF induction of E-selectin and VCAM-1 is confined to venular EC, whereas ICAM-1 is induced on all EC. Here, we compared the responses of Bcl-2-HUVEC to TNF before and after in vivo implantation. In newly formed gels (prior to implantation into mice), Bcl-2 HUVEC responded to TNF by upregulating E-selectin, VCAM-1, and ICAM-1 on all cells. However, when gels were implanted into SCID/beige mice for 30-60 days before s.c. injection of TNF (500ng), only a subset of Bcl-2-HUVEC upregulated E-selectin and VCAM-1, whereas all EC showed ICAM-1 upregulation (indicating all vessels were exposed to TNF). Subsequent anti-α-actin staining demonstrated that the subset of Bcl-2-HUVEC which upregulated E-selectin had a pattern of smooth muscle/pericyte investment consistent with venular differentiation. The correlation of morphologic features of vascular maturation and patterns of E-selectin or VCAM-1 upregulation suggests this model may be useful in analysis of microvascular differentiation and its relationship to adhesion molecule regulation.

0036

Total IgE serum level, but not total IgE blister fluid level; intensity of IgG and IgG4 skin deposits; and ECP serum and blister fluid levels, correlates with severity of active human bullous pemphioid

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It appears that human bullous pemphigoid (BP) at an initial stage is a substantially Th-2 mediated disorder. Also, there are reports suggesting that total IgE serum level correlates with the disease activity in human BP. The purpose of this study was to examine, using proper methods of statistical analysis, whether the action of IgE or IgG1 and IgG4 subclasses of IgG tissue-bound BP autoantibodies or eosinophils parallels most closely the entire active disease process in human BP. Nineteen patients with untreated confirmed BP at the blistering stage were enrolled in this study. A numerical grading system of active BP lesions was developed which included assessment of blisters, wheal-like lesions, pruritus/burning and area of skin involved. We evaluated total IgE and ECP serum and fresh (less than 24 hours old) blister fluid levels with fluoroenzymeimmunoassays, and the intensity of IgG and IgG4 dermal-epidermal junction (DEJ) deposits using direct immunofluorescence on saline-separated skin (SSDIF) with titration of FITC-conjugated anti-human IgG and IgG4 antibodies. Since IgG1 DEJ deposits were infrequently detected and generally weak and IgE DEJ deposits were detected in none of BP patients studied, despite using sensitive SSDIF, the analysis of their intensities could not be performed and their correlation studies were not done as planned initially. The statistical analysis using Spearman's rank correlation test revealed that, among laboratory parameters evaluated, only total IgE serum level correlated with the intensity of active lesions in human BP. Additionally, there was no correlation between the intensity of IgG and IgG4 DEJ deposits. Thus, it seems that IgE-mediated phenomena, albeit conceivably in a disease-nonspecific manner, might be more important than both IgG1/IgG4-mediated and eosinophil-mediated processes in pathogenesis of human BP.

0037

Inhibition of scratch behavior accompanied by reduced c-Fos expression in the spinal dorsal horn of histidine-decarboxylase gene knockout mice on contact dermatitis

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Patients with contact dermatitis suffer from strong itch sensation. In the present study, the role of chronic eczema-induced histamine on scratch behavior and neural conduction of itch sensation were investigated. Using L-histidine decarboxylase (HDC) gene knockout mice, which lack histamine in the body, chronic contact dermatitis was induced by repeated application of diphenylcyclopropenone on a hind paw. Scratching behavior was exclusively induced in HDC (+/+), whereas it was barely observed in HDC (-/-) mice. Expression of c-Fos was specifically up-regulated in HDC (+/+) mice in lamina I of the spinal dorsal horn. These results demonstrate that the afferent pathway of itch sensation, in a chronic contact dermatitis model, is transmitted by histamine below the lumber level, and connects with the CNS through lamina I of the spinal dorsal horn.

0038

Construction of naive human antibody library and generation of anti-keratin antibodies

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Antikeratin(AK) autoantibodies is proved exist in human serum and is a very important element in human immune system. For furthering the studies on AK autoantibodies, it is necessary to generate antikeratin human monoclonal antibodies. The aim of our present study was to construct a large naive human antibody library and then select anti-keratin antibodies from it. DNAs coding for κ chain and Fd fragments of human IgG and IgM repertoires were amplified by PCR from the peripheral blood mononuclear cells of non-immununized healthy donors and cloned into phagemid vector to construct respectively light and heavy chain libraries. And then a Fab fragment phage library was generated by combining the light chain and heavy chain genes. Human epidermal keratin was used as antigen to select antibodies from the library. By combination of light chain and heavy chain genes, five individual Fab fragment phage libraries each have approximately $0.5\sim4\infty10^8$ independent clones were generated and a total antibody library containing $1\infty10^9$ different clones was obtained. Three different specific phage antibodies against keratin were isolated by panning the library. One of them could express soluble Fab fragments and recognize *Mr* 48 000 keratin specifically. DNA sequencing of the variable regions demonstrated that the VH and VL of the antibody come from the human VH1 and Vκ2 families respectively. We conclude that the successful construction of the naive human phage antibody library create favorable conditions for preparing human monoclonal antibodies bypassing immunization. The produced anti-keratin antibody would be useful in the studies on keratin and AK autoantibodies.

0039

Ultraviolet light exposure suppresses contact hypersensitivity by abrogating endothelial intercellular adhesion molecule-1 up-regulation at the elicitation site

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Exposure to ultraviolet (UV) light-B radiation has the potential to affect humans. One of the most significant effects of UV radiation is systemic immune suppression that exacerbates a number of infectious diseases and permits the outgrowth of skin tumors. Hapten sensitization through UV-exposed skin induces systemic immune suppression, which has been experimentally demonstrated by inhibition of contact hypersensitivity (CHS). Although this UV-induced effect has been shown to be mediated by inhibition of the afferent phase of the CHS response, the UV effects on the efferent (elicitation) phase remain unknown. Since the elicitation phase is in part regulated by expression of cell adhesion molecules, UV-induced suppression of CHS was investigated in mice lacking either L-, P-, or E-selectin, or ICAM-1. Interestingly, UV exposure resulted in suppression of CHS responses in each of the selectin-deficient mice but not in the ICAM-1-deficient (ICAM-1-/-) mice. ICAM-1 deficiency did not affect the afferent limb since ICAM-1-/- mice were sensitized to hapten and had normal generation of hapten-specific suppressor and effector T cells. This UV-induced immunosuppression was associated with a lack of up-regulation of endothelial ICAM-1 expression and TNF-a production after Ag challenge at elicitation sites. Local TNF-a injection immediately before elicitation abrogated the UV-induced CHS inhibition, which was accompanied by increased endothelial ICAM-1 expression. TNF-a production was down-regulated by IL-10, a possible mediator produced by hapten-specific suppressor T cells that are generated by UV exposure. These results indicate that UV exposure inhibits CHS by abrogating up-regulation of endothelial ICAM-1 expression after Ag challenge at sites of elicitation.

0040

Epitopes and pathogenic activity of anti-desmoglein-1 in a subset of pemphigus foliaceus patients expressing only IgG1 autoantibodies

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Pemphigus foliaceus (PF) is an immunobullous diseases characterized by superficial blisters and IgG autoantibodies against the desmosomal antigen, desmoglein-1 (Dsg1). Previously, it was shown that IgG1 and IgG4 are the predominant isotypes of autoantibodies in these patients and only the latter isotype of anti-Dsg1 was capable of eliciting the experimental PF in neonatal mice. These results suggest that these two subclasses of autoantibodies may recognize different epitopes or express distinct tissue specificity. Based on our recent study, it is clear that IgG1 anti-Dsg1 recognizes both linear and conformational epitopes but fails to bind mouse skin. Anti-Dsg1 of the IgG4 isotype, on the other hand, only recognizes conformational epitopes, but is able to bind mouse skin. In this report, we described a subset of PF patients (4 of 52) who only expressed IgG1 anti-Dsg1 throughout their clinical courses. The sole IgG1 autoantibody profile in these patients was established by ELISA and direct and indirect immunofluorescent studies. Furthermore, we found that the IgG1 anti-Dsg1 from this subset of PF patients was able to reproduce the clinical, histological, and immunological features of the disease when injected into neonatal mice. Epitope analysis revealed that IgG1 anti-Dsg1 from these patients recognized both linear and conformational epitopes. Furthermore, using domain-swapped desmoglein proteins and recombinant Dsg1 fragments in ELISA and immunoblotting experiments, respectively, we found that the conformational epitopes of anti-Dsg1 autoantibodies are located at the N-terminus of Dsg1, whereas the linear epitopes are spread throughout the ectodomain of Dsg1. Our results further illustrate the complexity of autoimmune responses in patients with PF.

0041

Severe recalcitrant and ulcerative mixed connective tissue disease responding to intravenous immunoglobulin therapy

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High-dose IVIG has been used as a treatment modality in various diseases including dermatomyositis, lupus erythematosus, scleroderma and pansclerotic morphea. The presentation will discuss IVIG therapy for immune-mediated sclerosis syndromes and mechanisms of action. A 27-year old male presented with a 3-month history of widespread painful skin ulcers and 40-pound weight loss associated with prostration. Shortly after hospital admission, he developed acute Raynauds syndrome with dry gangrene formation involving all digits. His course was complicated by widespread herpes simplex infection, myositis, glomerulonephritis, interstitial lung disease and hemolytic anemia. Skin biopsy showed morphea. Pertinent laboratory findings included pancytopenia, elevated antinuclear and extractable nuclear antibodies and an elevated anti-U1 RNP. Initial treatment consisted of high-dose Solumedrol, Mycophenolate Mofetil, Trental, Norvasc and Heparin. Despite intensive wound care and aggressive immunosuppressive treatment for 3 months, he did not improve. High-dose IVIG (0.5 mg/kg weekly) was initiated. Within 3 weeks, his ulcerations healed, his systemic manifestations and overall status significantly improved. His elevated autoantibodies are unchanged to date. Mixed connective tissue disease is a rare disorder with overlapping features of scleroderma, systemic lupus erythematosus and dermatomyositis. Extensive skin ulcers as seen in our patient have rarely been described. The mechanism of action of IVIG is thought to be due to its ability to downregulate an IgG transport receptor and therefore accelerate the rate of IgG catabolism. Pathogenic autoimmune antibodies are likely neutralized by this mechanism. In addition, it reduces fibrosis in tight skin mice, probably by modulating TGF-beta and IL-4 expression. It inhibits T-cell proliferation, modulates cytokine production and interferes with complement activation. IVIG also improves opsonization and has antibacterial activity that may have contributed to the rapid healing of the cutaneous ulcers.

0042

Cholinergic stimulation inhibits pemphigus IgG-induced acantholysis and ameliorates clinical disease in a patient with pemphigus vulgaris

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Pemphigus vulgaris (PV) is an autoimmune, IgG autoantibody-mediated disease of mucocutaneous adhesion. Patients develop autoantibodies to adhesions molecules mediating intercellular cohesion of keratinocytes and to keratinocyte cholinergic receptors regulating cell adhesion. To determine whether a cholinergic agonist can abolish PV IgG-induced acantholysis, littermates of neonatal athymic nude mice were injected with PV IgG simultaneously with 0.04 mg/g body weight of carbachol (CCh). None of mice injected with PV IgG simultaneously with CCh developed skin lesions. To elucidate the mechanism of anti-acantholytic action of CCh, we measured the expression of adhesion molecules in monolayers of normal human keratinocytes incubated overnight in the presence of 0.25 mM of CCh, using semi-quantitative western blot and immunofluorescence. CCh caused elevation of the relative amount E-cadherin in keratinocytes ($P<0.05$), without changing that of plakoglobin ($P>0.05$). Since phosphorylation plays an important role in determining function of adhesion molecules, we also investigated the effects of CCh on pemphigus IgG-induced phosporylation of E-cadherin and plakoglobin, using the DJM-1 cells. PV IgG increased the phosphorylation levels of E-cadherin and plakoglobin, whereas in the presence of 0.5 mM of CCh this effect of PV IgG was attenuated. Pyridostigmine bromide (PBr), an acetylcholinesterase inhibitor, produced effects similar to those of CCh, which justified its administration to a patient with active PV that was resistant to treatment with systemic glucocorticosteroids. The PV patient treated with PBr (Mestinon), 360 mg per day, demonstrated direct strong interrelationship between the use of the cholinomimetic and the ability to maintain his pemphigus under control. Thus, novel anti-acantholytic therapies may be developed using cholinergic drugs.

0043

Elevation of IL-4 but not IFN-γ in the sera of patients with autoimmune chronic urticaria

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Chronic urticaria has been long considered a disease of unknown origin. Recent investigation has revealed that one third of patients with chronic idiopathic urticaria (CIU) have circulating functional autoantibodies against the high affinity IgE receptor, Fc εR I or IgE, suggesting that this disease may be diagnosed by a positive intracutaneous autologous serum skin test. In this study, we investigated whether the sera of patients with autoimmune chronic urticaria reflect predominance of a Th1 or Th2 phenotype. Using the ELISA technique, we measured IL-4 and IFN-γlevel in the sera of 40 chronic urticaria patients, including 15 patients with positive autologous serum skin test (ASST+) and 25 patients with negative autologous serum skin test (ASST-), and 26 healthy control subjects. We found that significant difference ($p<0.005$) existed of IL-4 levels in the sera of ASST+ chronic urticaria patients (6.05±2.74 pg/ml) vs. healthy controls (3.74±1.25 pg/ml). IL-4 levels were also elevated in ASST- chronic urticaria patients (5.46±3.02 pg/ml) compared to healthy controls ($p<0.01$). No significant differences in IL-4 levels were observed between ASST+ and ASST- chronic urticaria patients. No detectable levels of IFN-g were found in any sera of chronic urticaria patients and control subjects. We also observed a prevalent Th2 pattern in chronic urticaria patients. However, in autoimmune chronic urticaria patients there is not a prevalence of Th1 or Th2 cytokine pattern compared to ASST- chronic urticaria.

0044

An animal model of atopic dermatitis: lesional skin immunophenotyping delineates a CD 4 + T cell-predominated inflammatory infiltrate associated with up-regulation of adhesion molecules

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An animal model of chronic, pruritic, inflammatory skin disease has been developed in an epidermally expressed IL-4 Transgenic (IL-4-Tg) mouse line closely resembling human atopic dermatitis (AD) [J Invest Dermatol 2001; 117:977-83]. To define the in-situ inflammatory cell types and inflammatory mediators, immunofluorescence microscopy was performed on lesional and non-lesional (NL) skin of 15 PCR-confirmed IL-4-Tg mice and on skin of 15 age-matched Non-Tg mice. Statistical analyses performed, using a two-tailed Student t-Test at a 95% confidence level, showed a CD4+ T cells-predominated inflammatory infiltrate in lesional skin, a finding consistent with human AD. Every one of the dermal inflammatory cell types examined were significantly increased in number within lesional skin when compared with NL skin and Non-Tg mouse skin (all with a p value of <0.0001): CD11a+ bone marrow-derived leukocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells, CD23+ macrophages, P-selectin glycoprotein ligand (CD162)+ leukocytes, L-selectin (CD62L)+ activated leukocytes. In the lesional dermis the CD3+ T cells composed nearly 30% of CD11a+ leukocytes while the CD4:CD8 T cell subsets ratio was 2.5:1. In the lesional epidermis, a few CD4+ T cells (0.27/high power field (HPF)) and CD8+ T cells (1.83/HPF) were detected, and IA/IE+ antigen presenting cells were significantly increased when compared with NL skin ($p<0.0001$) and Non-Tg mouse skin ($p<0.0001$). Whereas adhesion molecules were undetectable in NL and Non-Tg mouse skin (except ICAM-1), they were highly expressed in lesional dermal blood vessels: ICAM-1 (5.47 vessels/HPF), VCAM-1 (1.96/HPF), E-selectin (4.41/HPF), P-selectin (7.91/HPF). Moreover, we also detected prominent lesional keratinocyte expressions of VCAM-1 and ICAM-1, a reported finding in human AD. Our data further support a close resemblance of our IL-4-Tg mouse disease model with human AD.

0045

Human beta-defensin expression in a new buccal epithelial tissue model

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Oral epithelia function as microbial barriers against bacteria and yeast that are resident in the oral cavity. These microbes can be categorized as non-pathogenic (commensal) and pathogenic (resulting in caries, gingivitis, and periodontal disease, etc.). The epithelium is actively involved in recognition and response to bacteria, setting into motion the innate immune responses that are an important part of periodontal health and disease. One epithelial response is the expression of antimicrobial peptides of the human beta-defensin (hBD) family. A new, three-dimensional model of the human buccal epithelium, based on normal human oral epithelial cells cultured in serum free medium, has been developed. Histologically, the tissue is 20-30 cell layers thick with cells becoming increasingly squamous toward the apical surface . No evidence of cornification is present and immuno-staining shows the expression of cytokeratin K13 in the suprabasal layers. Cytokeratin K4, the expression partner of K13, has also been detected by immuno-blotting. These features are characteristic of buccal epithelium. Additional immuno-staining revealed the presence of hBD-1 and the recently discovered hBD-3 in the suprabasal layers; hBD2 was not expressed. The hBD-1 and hBD-2 results parallel those of un-inflamed epithelia, however the hBD-3 result has not been previously reported. Based on these initial results, further investigation of the tissue model's hBD expression in unstimulated and bacterially challenged tissue is warranted.

0046

Avena Rhealba® inhibits arachidonic acid cascade, cPLA2 and COX2 expression in human keratinocytes. Interest in cutaneous inflammatory disorders

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Prostaglandins and leukotrienes represent an heterogeneous family of lipid mediators involved in inflammation and cell response regulation. During cutaneous inflammatory diseases, epidermal eicosanoid biosynthesis is dysregulated. Soothing oatmeal preparations seem to achieve special benefits on damaged skin and in patients suffering from eczema. Oatmeal extracts are present in cosmetic formulations such as emollients, skin softeners, moisturizers and skin protectors. Avena Rhealba®, a white oat species of Avena Sativa, is present in a variety of dermatological and anti-irritant products, and has beneficial effects on skin inflammation. The aim of the present study was to investigate the effects of Avena Rhealba® oatmeal extract on the cutaneous metabolism of arachidonic acid (AA) and eicosanoids, as well as on the expression of cPLA2 and COX2 in the human keratinocyte cell line HaCaT. Avena Rhealba® was found to inhibit the A23187-triggered increases in the levels of [3H]-arachidonic acid mobilization and of [3H]-labelled metabolites of the cyclooxygenase and 5-lipoxygenase pathways. A closer examination by enzymatic immunoassay EIA of the effects of Avena Rhealba® on 6KPGF1α, the stable metabolite of PGI2 released in cell supernatants, revealed the dose-dependent inhibition of this AA metabolite. Avena Rhealba® also decreased the A23187-induced cPLA2 and COX2 expression, as shown by cPLA2 immunodetection, and as revealed by RT-PCR analysis of cPLA2 and COX2 mRNA expression. Our results demonstrate the regulator properties of Avena Rhealba® with respect to inflammatory response showing, therefore, a real interest of Avena Rhealba® in cutaneous inflammatory disorders.

0047

Efalizumab modulates an array of adhesion-related and activation-controlling surface proteins on T lymphocytes and inhibits anti-CD3-induced T cell activation in adults with moderate to severe psoriasis

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The purpose of this study was to evaluate expression of adhesion molecules as well as numbers for various T cell subsets in peripheral blood lymphocytes from 13 subjects with moderate to severe plaque psoriasis treated with a humanized monocloncal antibody to CD11a (efalizumab). Flow cytometric analyses of peripheral blood mononuclear cells were conducted on Days 0, 14, 28, and 56 of treatment. Analysis of adhesion molecules revealed down-modulation of CD11a and CD18 on CD3+ lymphocytes persisting throughout the course of treatment. Similar to previously reported down-regulation of VLA-4, downmodulation was also observed for beta7 and, to some extent, for CD11b and L-selectin markers. There were no significant changes in the levels of CD11c, CD44, and cutaneous lymphocyte antigen. Down-modulation of the adhesion molecules is likely to contribute to the anti-adhesive effects of efalizumab. T cell numbers in the peripheral blood were increased during treatment. There was a marked increase in CD8+ memory cells, a T-cell population that is selectively increased in psoriatic lesions. This suggests re-trafficking or blocked tissue entry of an important disease-relevant cell population. Naive, but not effector CD8+ T-cell numbers were also increased, consistent with reduced entry into lymph nodes. No clear treatment-related pattern in Type 1 to Type 2 T cell ratio was noted. A decrease in the expression levels of CD4, CD8, CD2, and T-cell receptor alpha- and beta-chains on T cells was also observed, whereas CD3 expression stayed relatively constant. Using a T cell activation assay in which an antibody to CD3 induces increased expression of CD25 and CD69 activation markers, we observed that efalizumab treatment inhibits this response. This is the first demonstration in humans that inhibition of activation is a mechanism of action of efalizumab.

0048

Macrophage migration inhibitory factor expression by human dermal microvascular endothelial cells in response to thrombin and factor Xa

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Activated coagulation factors, aside from their critical role in thrombosis, also have inflammatory properties and these may be important during wound healing. Thrombin and factor Xa (FXa) are key proteases in the coagulation cascade and promote tissue repair responses. Both thrombin and FXa evoke a proinflammatory response in endothelial cells. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine, that has now been implicated in skin wound healing processes. This study investigated whether thrombin and FXa can upregulate MIF expression in human dermal microvascular endothelial cells (HDMEC). Thrombin increased MIF mRNA levels and stimulated the release of MIF protein in a time- and concentration-dependent manner by HDMEC. Thrombin-induced MIF mRNA expression was significantly reduced by thrombin-specific inhibitor hirudin. A synthetic thrombin receptor agonist peptide (TRAP-6) induced MIF mRNA expression; indicating that the protease-activated receptor (PAR)-1 may be important in mediating MIF expression. Exposure of cells to FXa also stimulated the production of MIF. The effects of FXa were blocked by antithrombin III, but not by hirudin, suggesting that FXa elicits these responses directly and not via thrombin. In this study, we have also demonstrated that PAR-1 and PAR-2 mRNA expression was upregulated by MIF treatment in HDMEC. In conclusion, these findings suggest that HDMEC may be a significant source of MIF in the inflammatory response to skin injury, and that MIF production is an important biologic consequence of PAR-1 and PAR-2 induction.

0049

Calcitonin gene-related peptide and adrenomedullin down-regulate cytokine-induced microvascular endothelial cell adhesion molecule expression

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Adrenomedullin (ADM) and the related neuropeptide calcitonin gene-related peptide (CGRP) are potent vasodilators and potentially capable of modulating biologic functions of epidermal and dermal cells. CGRP and ADM activate high-affinity G-protein coupled receptors consisting of receptor-activity modifying proteins (RAMPs) and a seven-transmembrane domain calcitonin receptor-like receptor (CRLR) with RAMP-1/CRLR as CGRP and RAMP2/CRLR as presumed ADM receptors. In this study we have tested the hypothesis that endothelial cell (EC) adhesion molecule (ICAM-1, VCAM-1) expression in human dermal microvascular EC (HDMEC) transfected with CGRP/ADM receptor components is modulated by CGRP or ADM, respectively. HDMEC isolated from human foreskins or cells of the EC line HMEC-1 were transfected with cDNA expression vectors for RAMP-1, RAMP-2 and CRLR with efficacies of 40 - 50%. Stimulation of EC overexpressing R1/CRLR or R2/CRLR with CGRP or ADM (0.01-1000 nM), respectively, resulted in a dose-dependent upregulation of intracellular cAMP indicating that the receptors are functional. Importantly, stimulation of HDMEC cells transfected with R1/CRLR or R2/CRLR with TNFα in combination with CGRP or ADM revealed a reduction of the TNF-induced expression of ICAM-1 and VCAM-1. However, neither transfection of HDMEC with the orphan CRLR nor R1 and R2 alone was sufficient to mediate full cAMP induction or reduction of TNFα-induced adhesion molecule expression, which confirms the notion that RAMP expression is essential for CGRP/ADM receptor function and pharmacology. Thus, these novel data suggest that CGRP and even more pronounced ADM by activating high-affinity CGRP and ADM receptors increase intracellular cAMP and are capable of antagonizing TNFα-induced EC ICAM-1 or VCAM-1 expression, which may be of importance for the regulation of leukocyte-endothelial cell interaction during cutaneous neurogenic inflammatory responses.

0050

The localization of autoantigens in SPD-type and IEN-type of IgA pemphigus—post embedding immunoelectron microscopic study

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IgA pemphigus is a rare autoimmune bullous disease characterized by IgA deposition at keratinocyte cell surfaces. Clinically and histologically, IgA pemphigus is divided into two major subtypes; subcorneal pustular dermatosis (SPD) type and intraepidermal neutrophilic IgA dermatosis (IEN) type. Using cDNA transfection and living cell immunofluorescence, we previously showed that, desmocollin 1, one of desmosomal cadherins, is the autoantigen in SPD-type of IgA pemphigus, but the autoantigen in IEN-type is still unclear. In the present study, we investigated antigen localization by using a post-embedding immunoelectron microscopy. We examined three sera each of SPD type and IEN type of IgA pemphigus. In the SPD-type, the majority of gold particles were observed in the intercellular spaces between keratinocytes at the desmosomal areas. No significant labeling was observed elsewhere. In the IEN-type, the gold particles were mainly observed in the intercellular spaces in the non-desmosomal areas. These results confirm that the IgA in the sera of SPD type IgA pemphigus react with extracellular domain of desmocollins. In contrast, the autoantigen for IEN type may not be desmosomal proteins. IEN type IgA pemphigus may be the first pemphigus group of autoimmune bullous dermatosis which reacts with non-desmosomal transmembranous protein.

0051

Th1 predominance of fluorouracil-induced discoid lupus erythematosus in TCR α chain knockout mouse

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Fluorouracil (FU) is well known to induce discoid lupus-like eruption in Japan. It is still obscure which cytokines are involved in the development of DLE. In this study, we established the murine model of FU-induced discoid lupus and studied the immunological characteristics of models of C57BL/6J (B6) mice, TCR α KO mice, and TCR δ KO mice treated with FU and UVB. FU was dissolved with PBS and intraperitoneal injection (0.2mg or 2mg) was performed every two day. UVB (200mJ/cm2) was irradiated to relative mice 5 times a week. Experimental group were as follows; PBS injection group, FU injection group, UVB irradiation group and the group of FU injection + UVB irradiation. TCR α KO mice treated with 0.2mg FU + UVB showed rapidly and frequently marked erythema at the neck and back with positive subepidermal IgG deposits. B6 mice treated with 2mg FU and UVB developed a slight erythema around the neck. B6 mice treated with PBS or FU alone had no changes. B6 mice treated with UVB and TCR α KO mice treated with FU showed high frequency of hyperkeratosis and perivascular lymphoid cell infiltration. CD 4+ T cells were predominantly observed in dermis of skin lesions. CD 8+ T cells also appeared slightly in epidermis and dermis in the skin lesions. In the skin lesions of TCR α KO mice treated with 0.2mg FU + UVB, Th1 type cytokine production such as IFN-γ and TNF-α were predominant by RT-PCR methods. However, anti-nuclear antibody of this group was low as well as other groups without eruption. We established drug-induced DLE model with using FU and UVB irradiation. We proposed that the association of Th1 type cytokine and T cells was pathogenic in the mechanism of drug induced DLE as well as idiopathic CCLE.

0052

The α-defensin HNP-1 inhibits superantigen-mediated T cell activation

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T-cells recognize antigen in the context of antigen-presenting HLA molecules. Antigens are typically up to 24 amino acids long and bind to the HLA binding groove in an extended conformation. To identify potential autoantigens in psoriasis, lesional epidermis from patients was removed using a dermatome. Subsequently, HLA class-II bound peptides were acid-eluted from keratinocytes. Peptide fractions obtained by HPLC were submitted to microsequencing by Edman degradation. Two dominant molecules were identified as α-defensins HNP-1 and -2. Their masses indicate unusual lengths of 30 and 29 amino acids, respectively. Utilizing molecular modelling, an HLA binding supermotif can indeed be identified. Bulky" peptides could prevent a bridging between HLA molecule and T-cell receptor, which is a prerequisit for T-cell activation by bacterial superantigens. To test this hypothesis, T-cells were incubated with staphylokokkal superantigens SEA and SEB in the presence of HNP-1. In agreement with the hypothesis, a dose-dependent inhibition of the expression of activation markers as well as cytokine secretion was observed. To date, defensins have only been isolated from HLA class-II molecules of cell types expressing them upon stimulation, e.g. in inflammation. Defensins are capable of out-competing antigen (Blood 2000, 95: 2890) and of blocking superantigen-mediated T-cell activation. Hence, they might play an important role during down-regulation of inflammation. Given the function of superantigens as triggers for psoriasis, this mode of action could also be explored therapeutically.

0053

VEGFR-2 antibody accelerates disease activity in lupus-prone NZB/W F1 mice

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Inhibition of angiogenesis has shown significant promise in the treatment of solid tumors. Angiogenesis inhibitors have also demonstrated benefit in certain inflammatory and autoimmune diseases, including psoriasis. Vascular endothelial growth factor (VEGF) is one of the main mediators of angiogenesis. In Systemic Lupus Erythematosus (SLE), levels of VEGF have been correlated with some clinical and laboratory parameters, as well as overall disease activity. To further examine the role of VEGF in SLE we examined the effect of anti-VEGF receptor-2 (VEGFR2) antibody treatment using the murine model for SLE (the NZB/W F1 mouse). We hypothesized that anti-VEGFR2 would delay the onset of clinical disease and reduce mortality. Anti-VEGFR2 antibody was administered IP to NZB/W F1 mice (beginning at 6-8 weeks of age) every three days. After 12 weeks of treatment, the anti-VEGFR2 antibody-treated group displayed decreased renal function and moderate (2+) proteinuria, with severe (3+) proteinuria by 18 weeks. In contrast, the control group demonstrated trace proteinuria at 12 weeks, with 1+ - 2+ proteinuria by 18 weeks. Histopathologic examination of kidneys demonstrated advanced membranous glomerulonephritis at 12-14 weeks. These changes, although present, were significantly less prominent in the PBS control group. Mortality at 18 weeks was 76%, compared to 21% in the control mice. Thus, anti-VEGFR2 antibody treatment accelerated the severity of renal disease and mortality rate in this murine model of SLE. If these findings can be extrapolated to human studies, our findings could have profound implications on anti-angiogenesis therapies in patients with underlying autoimmune disorders.

0054

Anti-inflammatory effect of peroxisome proliferator-activated receptor agonists through the suppression of nuclear factor κB in human epidermal keratinocytes

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family. We showed that the agonists to PPAR-α and -γ strongly inhibited interleukin (IL)-1β-induced IL-8 synthesis by cultured normal human epidermal keratinocytes (NHEKs) at mRNA and protein levels. To clarify the mechanisms involved in this anti-inflammatory process, we examined the effect of PPAR-γ agonists on nuclear factor κB (NF-κB) and IκB, a ubiquitous transcription factor and its inhibitory molecule, by immunohistochemical staining and electrophoretic mobility shift assay. A PPAR-γ agonist, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), enhanced the expression of IκB in the cytoplasm. PGJ_2 inhibited the nuclear translocation of NF-κB in IL-1β-stimulated NHEKs. Troglitazone, another PPAR-γ agonist, did not show the inhibitory effect on NF-κB activation. These findings indicate that the anti-inflammatory effect of PPAR-γ agonists takes place through at least two pathways, namely NF-κB-dependent and NF-κB-independent pathways.

0055

CD4$^+$ T cell-associated pathophysiology critically depends on CD18 gene dose effects in a murine model of psoriasis

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Previously, a CD18 hypomorphic polygenic PL/J mouse model with severe reduction of CD18 (β_2-integrin) of 2-16% of the wild-type results in the development of a psoriasiform skin disease. To investigate the interrelationship of reduced CD18 gene dose effects on T cells in the pathogenesis of this dermatitis, immunostainings and *in vivo* depletion experiments were performed. CD4$^+$ and CD8$^+$ T cells were significantly increased in the skin of affected CD18 hypomorphic compared to wild-type mice. Depletion of CD4$^+$ T cells, but not of CD8$^+$ T cells, resulted in a complete resolution of the severe psoriasiform dermatitis, indicating a central, CD8$^+$ T cell independent role of CD4$^+$ T cells in the pathogenesis of the disease. In contrast to the CD18 hypomorphic PL/J mice, CD18 null mutants of the same strain did not develop the psoriasiform dermatitis. This is in part due to a lack of T cell emigration from blood vessels of the skin, as after oxazalone challenge, allergic contact dermatitis could be induced in CD18 hypomorphic and wild-type mice but not in CD18 null mutants. Hence, 2-16% of CD18 gene expression is obviously sufficient for T cell emigration driving an inflammatory phenotype in CD18 hypomorphic PL/J mice. Our data suggest that the pathogenic involvement of CD4$^+$ T cells depends on a gene dose effect with reduced expression of the CD18 protein in the underlying mouse model. This inflammatory skin model may have general relevance to polygenic human inflammatory diseases.

0056

Th1 and Th2 cytokine mRNA profiles in a murine scleroderma model

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We have investigated T helper cell (Th1 and Th2) cytokine mRNA levels in skin from a murine model for scleroderma, sclerodermatous graft versus host disease (mScl GVHD). Bone marrow and spleen cells from B10.D2 (H-2^d) mice were transplanted to lethally irradiated BALB/cJ (H-2^d) mice. The transplanted mice developed cutaneous monocyte/macrophage and T cell infiltrates, skin thickening, and lung fibrosis, while syngeneic transplanted mice showed no disease. We analyzed a panel of Th1 (IFN-γ, IL-2) and Th2 (IL-5, IL-6, IL-10, IL-13) cytokines by assessing the levels of skin total RNA with semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). IFN-γ, IL-2, and IL-10 mRNAs were up-regulated during development of skin fibrosis consistent with both Th1 and Th2 profiles in this animal model for chronic scleroderma. This result is similar to the mixed Th1/Th2 profile in human scleroderma, in which the cytokine profile may be a useful serologic marker for severe disease. Of interest, cytokines (IFN-γ and IL-10) that inhibit collagen production by fibroblasts in vitro were paradoxically elevated in vivo as fibrosis developed, suggesting a complex immunomodulatory environment in early fibrosing disease.

0057

Regulatory role for macrophage migration inhibitory factor in TNCB-induced allergic contact dermatitis

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The cytokine macrophage migration inhibitory factor (MIF) has emerged to be an important regulator of the inflammatory response and is critically involved in the development of septic shock, arthritis, hepatitis, and glomerulonephritis. Although MIF expression was limited to the basal layer in normal human epidermis, it was reported that MIF expression was markedly up-regulated in various skin conditions such as atopic dermatitis, psoriasis, and UVB-induced dermatitis. In this study, we sensitized 10-week old C3H female mouse by 2,4,6-trinitrochlorobenzene (TNCB). Western-blot analysis showed that highest expression level of MIF in the ear skin was seen at 1 h after the elicitation and the up-regulation continued to 6 h in comparison with the basal level (0 h). We also measured dopachrome tautomerase activity of MIF using the same samples and observed that MIF enzyme activity was elevated in the same time-course manner as shown in western-blotting.

0058

Saccharomycopsis ferment filtrate inhibit lipopolysaccharide-induced inflammatory effect

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Yeast extracts have been used extensively to perform several beneficial biological activities; however, the mechanism is still undefined. In the present study, we provided evidences to demonstrate that saccharomycopsis ferment filtrate (SFF) is able to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in a concentration-dependent manner without notable cytotoxic effects on cells by MTT assay. A decrease in NO production by SFF was associated with an induction of heme oxygenase-1 (HO-1) protein expression and an inhibition in iNOS protein by Western blotting. Hemin, a HO-1 inducer, dose dependently induced HO-1 protein, was accompanied by a suppression in iNOS protein expression. Furthermore, addition of hemin stimulated SFF-induced HO-1 protein, at the same time, potentiated the inhibitory effects of SFF on LPS-induced NO production and iNOS protein expression. These results provided an interesting correlation between HO-1 induction and NO inhibition, and predicted SFF inhibited LPS-induced NO production might be mediated by HO-1 induction.

0059

Involvement of apoptosis in bleomycin-induced scleroderma

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We previously established a mouse model for scleroderma induced by repeated injections of bleomycin (BLM). In this study, we examined the possible involvement of apoptosis in the development of dermal sclerosis induced by BLM. TUNEL analysis showed positive staining in keratinocytes and infiltrating mononuclear cells following BLM treatment. TUNEL-positive fibroblasts were also scattered, but not endothelial cells. DNA fragmentation was detected at 3 and 4 weeks after BLM exposure. Fas and FasL expression was upregulated at protein and mRNA levels. Expression as well as activity of caspase-1 and -3 was also increased following BLM exposure. These findings suggest an important role of apoptosis in the pathogenesis of bleomycin-induced scleroderma.

0060

Immunosuppression by induction of heme oxygenase 1: *in vitro* mechanism and effect in models of allergic contact dermatitis

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The strong antiinflammatory and immunosuppressive potency of the stress protein heme oxygenase 1 (HO-1) has been proven in many models of inflammatory injury and autoimmunity. Hitherto, the underlying immune mechanisms have been poorly understood, and no data are available about the effect of HO-1 induction in models of inflammatory dermatoses. In our study, the potent HO-1 inducer Cobalt-Protoporphyrin IX (Co-PP) strongly suppressed T-cell proliferation in mixed lymphocyte reaction (MLR). This inhibition was due to a depression of antigen-presenting capacity, accessory molecule expression, and stimulatory cytokine secretion of monocytes. In vivo, dendritic cells (DC) represent the main antigen-presenting cells. Remarkably, the HO-1 inducer Co-PP led to an almost complete inhibition of the differentiation, maturation, and function of monocyte-derived dendritic cells. So, a strong decrease of the expression of DC markers (CD1a, CD83) and accessory molecules (HLA-DR, CD86) was observed accompanied with an increase of the expression of phagocytic receptors (CD14, CD16). Together these changes indicated a switch of the DCs to an immature and macrophagic phenotype. In fact, the antigen-presenting capacity of Co-PP-treated monocyte-derived DCs was strongly diminished in MLR with allogeneic CD4+ T-cells. The in vivo immunosuppressive activity of HO-1 induction was tested in DNFB (Type 1) and TMA (Type 2) models of ear inflammation in mice. Treatment with the HO-1 inducer Co-PP at 24h before challenge dose-dependently depressed ear edema, infiltration, and cytokine levels. Importantly, Co-PP also strongly suppressed T-cell-dependent inflammation when applied around sensitisation to prevent the initiation of the specific immune response. We hypothesise that the inhibition of DC maturation and function is the crucial mechanism for this immunosuppressive activity of HO-1 induction which may represent a novel approach for the treatment of T-cell mediated dermatoses like psoriasis, contact eczema, and atopic disease.

0061

Human bullous pemphigoid antigen 2 (hBPAG2) in transgenic murine skin elicits immune responses in wild type (wt) mice that show fidelity to those seen in patients with bullous pemphigoid

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BP is a subepidermal blistering disease characterized by IgG autoantibodies against BPAG2, a type II transmembrane collagen that promotes adhesion of basal keratinocytes to epidermal basement membrane (BM). To model experimental immune responses against this protein, we produced C57BL/6 Tg mice expressing hBPAG2 in murine epidermis under the control of a human keratin 14 promoter. Monoclonal and polyclonal antibodies identified hBPAG2 in the epidermal BM of Tg mice; post-embedding immunogold electron microscopy of cryofixed and cryosubstituted Tg skin showed that the carboxyl terminus of hBPAG2 was appropriately localized beneath hemidesmosomes within the superior aspect of the lamina densa. Grafts of Tg skin placed on gender-matched, syngeneic wt mice elicited IgG that bound: 1) human and Tg epidermal BM; 2) BPAG2 extracted from human keratinocytes; and 3) the NC16A domain of hBPAG2. Production of such IgG in naive mice was prompt (detectable within 17±3 days), robust (titer > 1280), and durable (present >270 days). Lymph nodes and splenocytes from wt mice grafted with Tg skin contained T cells that displayed antigen-specific proliferative responses to the NC16A domain of hBPAG2. Anti-hBPAG2 IgG did not develop in MHC class II -/- mice grafted with Tg skin, indicating that cognate interactions between T cell receptor/peptide-class II MHC determinants were crucial for antigen-specific IgG production in this model. Grafts of hBPAG2 Tg skin on syngeneic wt mice elicited B and T cell responses against the same portion of hBPAG2 that is bound by autoantibodies and T cell lines from patients with BP. This Tg approach has yielded an experimental model in which immune responses to hBPAG2 can be elicited, characterized, and potentially modulated or blocked in vivo.

0062

Cleavage of desmosomes occurs without keratin retraction *in vivo* in pemphigus vulgaris model mice

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Pemphigus vulgaris (PV) is an autoimmune blistering disease caused by IgG autoantibodies against desmoglein3 (Dsg3). The exact mechanism of blister formation after IgG autoantibody binding is still unknown and three mechanisms are proposed: 1) keratin retraction, 2) specific depletion of Dsg3 from desmosomes (DMs) and 3) direct inhibition of the adhesive function of Dsg3. Previously, we have demonstrated that Dsg3 in DMs is fully accessible in vivo by IgG autoantibodies and that Dsg3 is not apparently depleted from DMs in vivo by immunoelectron microscopic studies with PV model mice. In this study we focused on acantholytic and non-acantholytic lesions of PV model mice by immunoelectron microscopy to elucidate the molecular mechanism of blister formation. A lot of half-split DMs were found at the blister roof (basilar surface of suprabasilar cells) as well as floor (apical surface of basal cells) in acantholytic area of PV model mice, and the half-split DMs were decorated with IgG and their plaques were accompanied with keratin filament insertion. Desmoplakin was detected between keratin filaments and attachment plaques of the half DMs as much as observed in DMs in non-acantholytic areas. A few degrading DMs, which were decorated with IgG and lacked connection of keratin filaments, were found at the lateral surface, but not at the apical surface, of the basal keratinocyte in both acantholytic and non-acantholytic lesion. In those degrading DMs, desmoplakin was localized at the end of keratin filaments in the cytoplasm indicating keratin filaments retract from DMs with desmoplakin attached. These results indicate that the cleavage of DMs takes place without keratin filament retraction from the attachment plaques in vivo. Together with our previous finding that Dsg3-/- mice form a lot of half-split DMs in the acantholytic area, our current findings strongly suggest that blister formation in PV is mainly induced by loss of function of the desmosomal Dsg3 in vivo but not by keratin retraction.

0063

Level of vascular endothelial growth factor is elevated in atopic dermatitis

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Atopic dermatitis (AD) is a chronic inflammatory dermatosis occurring mostly on the basis of atopic diathesis. The characteristics of AD skin lesions are continuous erythema and recurrent exudative change due to hyperpermeability. Vascular endothelial growth factor (VEGF), of which six distinct splice variants (VEGF 121, 145, 165, 183, 169, 206) have been described, plays an important role in the vascular permeability and angiogenesis associated with many malignant tumors. Recently, in inflammatory dermatosis, such as psoriasis, expression of VEGF was found to be enhanced in the epidermis of the lesions. In this study we investigated the level of VEGF in the lesional scales of patients with AD to know an involvement of VEGF in the pathogenesis of AD. The contents of VEGF were measured using an ELISA kit and the molecular mass of VEGF was determined by western blot analysis of proteins immunoprecipitated with agarose conjugated anti-VEGF antibody. As a result, the level of VEGF in the lesional scales was 202±120 pg/mg scale, n=23, and was significantly high compared with that obtained from normal scales of the patients(10.3±5.6 pg/mg scales, n=23). The result suggests that VEGF is involved in the inflammatory process of AD and blockade of the action may be a new therapeutic strategy for AD. On immunoblotting, two bands, 20 and 15 kDa were detected possibly corresponding to VEGF165 and 121, but not in normal scales.

0064

The immunofluorescence pattern induced by sera from patients with palmoplantar pustulosis

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PPP patients have an increased prevalence of autoimmune diseases. We have recently reported that 47% of sera (n=45) from patients with PPP give an immunofluorescence (IF) of the papillary endothelium in palmar skin from healthy subjects. The aims of this study was a) to screen a larger group of PPP patients (n=89) with regard to IF in the palmar skin in order to confirm our first results; b) to screen other skin areas (elbow, scalp, gluteal region) for IF and c) to screen non-dermal tissues (thyroid gland, parathyroid gland, pancreas, liver, kidney, intestine) for IF with indirect immunofluorescence technique. All specimens were from healthy subjects and other normal tissue specimens had been removed in conjunction with surgery. Furthermore, PPP sera were screened for the presence of bands specific for PPP with Western blot on homogenates from palmar skin from a healthy smoker. Forty-seven percent of the 89 PPP sera gave an IF of palmar endothelium (from healthy donors). IF was unchanged when sera were taken from the same patient at several occasions over a period of up to 5 years (n=8). Sera which induced an IF in palmar skin also gave an IF in elbow and gluteal skin but only of the inner surface of the vessel and there was no chain-like pattern as in the palm. The endothelium in the scalp was, however, negative. IF positive sera also induced an IF of endothelium in the parathyroid gland and also -but much less pronounced - in the thyroid gland, pancreas, liver, kidney, intestine. For comparison sera from healthy subjects and from patients with hand eczema were also screened and gave no IF. With Western blot PPP sera showed reactivity against 4 different (not identified) antigens from palmar skin with molecular weights from 33 to 43 kDa. None of these bands were observed with control sera. The immunofluorescence results indicate that PPP may be a systemic autoimmune disease involving the endothelium.

0065

Differentiation of subepidermal autoimmune bullous diseases in laser scanning confocal microscopy

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We studied 50 patients fulfilling clinical and immunelectron microscopic criteria for bullous pemphigoid-BP (38) cicatricial pemphigoid-CP (7) and epidermolysis bullosa acquisita (5). Laser scanning confocal microscopy (LSCM) technique was used to perform three dimentional reconstruction (3D) of basement membrane zone (BMZ) as well as to determine the localization of the in vivo bound IgG at the BMZ in patients skin in compare to the localization of lamina lucida and lamina densa markers. In vivo bound IgG were visualized by labeling with FITC-conjugated goat anti-human IgG antibody, whereas BMZ markers were labeled with anti-mouse Cy5-conjugated antibodies. Excitation of FITC and Cy5 was simultaneously performed with laser lines of 488 nm and 637 nm respectively. In BP in vivo bound IgG was localized on the epidermal side of laminin-5 and co-localized with beta4 -integrin. In CP cases IgG was in vivo bound at the dermal-epidermal junction between localization of laminin-5 and collagen IV. In EBA in vivo bound IgG was found to be present on the dermal side of collagen IV. 3D reconstruction of various regions of BMZ was performed in 20 micrometers thick perilesional patients skin with BP (3), CP (3) and EBA (3). A stack of 40 optical slides was collected automatically every 0.5 micrometers step with the scan speed 166Hz by computer program integrated with LSCM. Our studies revealed only numerous invaginations of BMZ on the level of lamina lucida and lamina densa in BP and CP. In EBA we noticed discontinuous staining on the level of sublamina densa region and large clumps of collagen VII vertically orientated to the BMZ extending into the deep dermis. LSCM allows differentiation of autoimmune subepidermal bullous diseases. These methods are of special value in those cases in which circulating autoantibodies are not detectable.

0066

Successful treatment of autoimmune urticaria with a combination of plasmapheresis and intravenous corticosteroid

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A 66-year-old woman presented with a 5-months history of urticaria unresponsive to H1-antagonists. Histological examination of the lesion revealed no sign of vasculitis. No significant abnormalities were found on physical examination, or laboratory examination including urinalysis, complete blood cell count, serum electrolytes, liver function tests, creatinine, blood urea and nitrogen and total serum IgE. Specific IgE antibodies in the serum were not detected. Intracutaneous testing of autologous serum revealed a 12 x 10-mm wheal and flare reaction. Histamine-releasing test with the serum showed 28% histamine release from basophils of a healthy donor, and this was significantly enhanced (81%) when IgE on the cells were stripped by lactate-treatment, suggesting the anti-FceRI-alpha antibodies (competitive type) in the serum. Theurticaria was partially reactive to the intravenous corticosteroid but the wheal was refractory. Then the patient was treated with plasmapheresis (4 times) and intravenous immunoglobulin (0.4g/kg/day for 5 days) followed by intravenous corticosteroid (500mg/day for 3 days). After the treatment there was a complete remission for more than 3-months. Repeat intracutaneous testing and histamine-releasing test to autologous serum became negative, indicating an abrogated autoantibody response. The result suggests an involvement of autoantibodies in the urticaria and efficacy of a combination of plasmapheresis and intravenous corticosteroid in this case.

0067

BDCA-2 and CD91 are co-expressed by plasmacytoid dendritic cells in skin lesions of subacute cutaneous lupus erythematosus, discoid LE, and dermatomyositis

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INF-α/B-producing, CD11c(-), CD123[high] antigen-presenting PDC might play a role in the pathogenesis of both the systemic and cutaneous features of LE. We have begun studies to further characterize the phenotype of this cell and to determine its specificity for different clinical types of cutaneous LE as well as related autoimmune skin disorders. Lesional biopsies were collected from patients with SCLE (n=6), DLE (n=1), and dermatomyositis (DM) (n=1). Using a frozen section immunofluorescence (IF) microscopy, we confirmed that PDC are present in a patchy distribution within the inflammatory infiltrate near the dermal-epidermal junction of SCLE, DLE, as well as DM skin lesions. No such reactivity was observed in 6 biopsies from normal human newborn foreskin. Using a multicolor IF technique, we have also demonstrated for the first time that PDC in cutaneous LE and DM skin lesions also express BDCA-2, a novel PDC-specific type II C-type lectin that mediates antigen capture and inhibition of INF-α/B production. In 5/6 SCLE patients, CD91 (the α-2-macroglobulin receptor) was found to be co-expressed with BDCA-2 on PDC. These preliminary results suggest that BDCA-2(+), CD91(+) PDC are present in the inflammatory infiltrate of LE-specific skin lesions. However, this cell type is not specific for cutaneous LE in that it is also present in other autoimmune inflammatory dermatoses such as DM and psoriasis (personal communication, Brian Nickoloff, M.D, Ph.D.). As excessive production of INF-α/B is thought to play a central role in the immunoregulatory abnormalities of LE, modulation of the BDCA-2 signaling pathway in PDC might provide a therapeutic strategy for cutaneous LE and perhaps other autoimmune skin diseases. In addition, the co-expression of CD91 by PDC in cutaneous LE lesions makes this cell type a candidate for an autoantigen presenting cell since CD91 is known to mediate the uptake of heat shock protein-antigenic peptide complexes and cross presentation of such peptide antigens.

0068

IgG4 is the predominant IgG subclass in gestational pemphigoid

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Past evidence indicates the linear deposition of C3 along basement membrane zone of skin (BMZ) and presence of circulating HG factor as the hallmarks for gestational pemphigoid. This study was designed to evaluate the effect of IgG subclass distribution on the result of direct immunofluorescence (DIF) of specimens from patients with gestational pemphigoid. Ten available skin biopsy specimens were obtained from our tissue banks, and subjected to indirect immunofluorescence (IIF) to detect the presence of IgG as well as its subclasses along BMZ of skin. All 10 specimens had strong C3 linear staining, but only 5 of them with trace amount of IgG staining along BMZ under DIF. All 10 specimens were positive for IgG deposition in IIF. On IIF for IgG subclasses, all 10 specimens were positive for IgG4, while 7 for IgG2, 5 for IgG1 and 4 for IgG3. The results of this study indicates that IgG can be detected on the skin specimens of patients with gestational pemphigoid, if IIF is employed, and IgG4 is the predominant IgG subclass involved.

0069

Role of Fc receptors in autoimmune bullous pemphigoid

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Bullous pemphigoid (BP) is a subepidermal bullous dermatosis of the elderly. BP patients exhibit circulating and tissue-bound autoantibodies directed against the hemidesmosomal antigens BP180 and BP230. Using an animal model of BP, which involves the injection of neonatal BALB/c mice with rabbit anti-murine BP180 (mBP180) antibodies, we have shown that subepidermal blistering triggered by anti-mBP180 IgG depends upon complement activation, mast cell degranulation, and neutrophil infiltration. The molecular mechanism for neutrophil activation in experimental BP has previously remained unknown. In this study, we tested the hypothesis that infiltrating neutrophils are activated through the molecular interaction between Fc receptors (FcR) on neutrophils and the Fc domain of pathogenic anti-BP180 IgG. We found that FcgRI and III double-gene knockout mice and FcgRIII single-gene knock out mice are resistant to experimental BP. In vivo deposition of pathogenic IgG and complement component C3 were detected in the skin of the FcgR-deficient mice, but the mice showed no clinical or histological skin lesions. FcR blocker specific for FcgRII and III abolished the BP phenotype in wild-type mice injected with pathogenic IgG in vivo and inhibited activation of wild-type neutrophils stimulated by pathogenic IgG in vitro. Pathogenic IgG activated wild-type neutrophils to secrete neutrophil elastase and gelatinase B, but failed to stimulate FcR-deficient neutrophils to release these proteolytic enzymes. In addition, we found that the pathological function of anti-mBP180 IgG depends entirely on its Fc domain; F(ab')2 fragments of IgG had no pathogenic activities in vivo or in vitro. Results from this study establish that FcgRIII plays a critical role in the activation of infiltrating neutrophils, which in turn release proteolytic enzymes resulting in dermal-epidermal blister formation. These findings give us novel insights into the immunopathology of BP and other IgG- and neutrophil-mediated autoimmune diseases and may help us to develop new therapeutic strategies for these diseases.

0070

Extracellular matrix protein 1 is a target autoantigen in lichen sclerosus

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Lichen sclerosus (LS) is a chronic mucocutaneous scarring disease of unknown etiology. However, previous studies have found associations between LS and specific HLA subtypes as well as other autoimmune disorders, raising the possibility that the pathogenesis of LS might be explained, in part, by development of autoantibodies to an as yet unidentified target antigen in skin and mucous membranes. One putative autoantigen is the glycoprotein extracellular matrix protein 1 (ECM1). The rationale for this is based on histological similarities between LS and the genetic disorder lipoid proteinosis that results from mutations in the ECM1 gene. In this study, we analyzed serum autoantibody profiles in patients with ano- and extra-genital LS by immunoblotting (human epidermal and dermal extracts) and detected circulating IgG autoantibodies in 57/78 samples (73.1%). Specific immunoreactivity to ECM1 was confirmed in these cases by generating a full-length GST-fusion protein. Positive reactivity to the recombinant protein was also detected in ano-genital lichen planus (16/44, 36.4%), but to a lesser extent in systemic lupus erythematosus (3/45, 6.7%), systemic sclerosis (1/22, 4.5%), bullous pemphigoid (3/40, 7.5%) and normal control samples (1/35, 2.9%). Indirect immunofluorescence using affinity purified IgG fractions from LS sera revealed intense cytoplasmic staining in the basal keratinocyte layer in a similar distribution to labeling with a polyclonal anti-ECM1 antibody raised against a C-terminal oligopeptide. The positive staining was blocked by pre-absorption of the affinity purified IgGs with excess recombinant ECM1 protein in a dose-dependent manner, but not with GST alone or with recombinant BPAG2 NC16A protein. These data provide evidence for a specific humoral immune response to ECM1 in LS, as well as some cases of lichen planus, and provide new clues to disease pathogenesis.

0071

Objective *in vivo* quantification of edema

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Edema is associated with the expression of skin inflammation. It is characterized by an increase in extracellular water in the dermis leading to swelling. It frequently present together with vascular reactions. Edema and erythema are typically graded visually. In this study we investigated the objective assessment of histamine-induced edema in vivo with novel optical methods. Histamine was delivered iontophoretically from aqueous solutions at concentrations of (0-0.1% w/v) to the volar forearms of volunteers. The current delivery was 0.5 mA for 30 seconds. Measurements were acquired at the maximum of the reactions (20-30 min after treatment). The erythema and edema became evident at 15 min and lasted for 1-2 hours. The erythema intensity and the area of involvement were quantified by diffuse reflectance spectroscopy (DRS) and by hyperspectral imaging in the visible. The change in volume, the total area of involvement and the maximum height of the edema response were measured by 3D imaging. Extracellular water accumulation in the dermis at the edematous area was quantified by hyperspectral imaging in the NIR range. All measured parameters (oxy-hemoglobin concentration, extracellular water accumulation, swelling volume) demonstrated dose dependence. The skin responses ranged from perifollicular erythema at low doses to intense erythema plus edema extending beyond the treated site for high doses. The erythema intensity as measured by DRS increased linearly up to 0.001% of histamine. The range of the measured edema height at maximum was 0.16-0.96 mm and the range of the measured edematous volume was 0.3-140 mm3. Extracellular water accumulation was documented by spectral imaging of the treated site at 970 nm referenced to 900 nm. In summary, we have demonstrated that parameters that characterize cutaneous edema, accumulation of extracellular water in the dermis, local increase in the volume of the tissue, and erythema, can be quantified accurately in vivo non-invasively by optical methods. Such methods can be applied to effectively measure the extent of an edematous response as well as the efficacy of treatments.

0072

Tolerance escape of autoreactive B lymphocytes against desmoglein 3 in transgenic model mice

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Pemphigus vulgaris (PV) is an anti-desmoglein3 (Dsg3) IgG mediated autoimmune blistering disease. It remains unclear how self-tolerance against Dsg3, a peripheral antigen, is broken to trigger the disease. The purpose of this study is to understand the mechanism of B cell tolerance against Dsg3 using anti-Dsg3 autoantibody producing transgenic mouse model. We isolated a monoclonal IgG antibody AK7 that recognizes native mouse Dsg3 in vivo from PV model mice produced by adoptive transfer of Dsg3-/- splenocytes into Rag2-/- mice. cDNAs for heavy and light chain variable regions were isolated from the AK7 hybridoma and introduced into transgenic constructs containing IgM backbone. We then generated IgM transgenic mice (AK7-LH1) in C57BL/6 mice expressing endogenous Dsg3. Self tolerance are considered to be established by deletion or inactivation of self-reacting lymphocytes. In AK7-LH1 mice, AK7-IgM expressing B cells were detected in the bone marrow where most of self-reactive B cells against central antigens are eliminated. Surprisingly, however, AK7-IgM expressing B cells were also observed in the peripheral lymphoid organs, including spleen and lymph nodes. More than 90% of heavy-chain transgene positive B cells recognized recombinant Dsg3. Dsg3 recognizing B cells were as much as 13.7±2.5% of total splenocytes (n=5). Furthermore, AK7-IgM was readily detected in circulation and the titers of anti-Dsg3 IgM were over 10 times higher than those of PV model mice. There was in vivo IgM deposition on keratinocyte cell surfaces of mucosal epithelia where Dsg3 is expressed, although there was no apparent PV phenotype in these mice. Thus, Dsg3-reacting B cells were not deleted nor inactivated in our transgenic mouse model. These findings indicate that Dsg3-reactive B cells, at least partially, escape from self-tolerance, and shed light on a new aspect of mechanism for peripheral tolerance.

0073

Identification of the hRDH-E2 gene, a novel member of the SDR family, and its increased expression in psoriatic lesions

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To identify novel psoriasis-associated genes, we focused on several ESTs (expressed sequence tags) whose expression was predominantly increased in the affected skin in patients with psoriasis vulgaris, as assessed by microarray assay. In the present study, a full-length cDNA corresponding to one of those ESTs (AI440266) was isolated by screening of cultured human keratinocyte cDNA libraries. This cDNA has an open reading frame of a 309-amino-acids protein, sharing significant homology to one of the short-chain alcohol dehydrogenase/reductase (SDR) family that can catalyze the first and rate-limiting step that generates retinaldehyde from retinol. So, this gene was designated as hRDH-E2 (human epidermal retinal dehydrogenase 2). The hRDH-E2 gene has a single functional copy on chromosome 8q12.1, spanning approximately 20 kb with seven exons. The deduced amino acid sequence contains three motifs that are conserved in the SDR family. Qualitative RT-PCR demonstrated that the mRNA levels of hRDH-E2 were significantly elevated in the affected skin in psoriasis patients as compared to the unaffected skin in patients and the normal skin in healthy individual. These results suggest that hRDH-E2 may be involved in the pathogenesis of psoriasis through its critical role in retinol metabolism in keratinocyte proliferation.

0074

Fcα-dependent granulocyte activation is required for blister induction by IgA1 autoantibodies to the cleaved ectodomain of BP180/Type XVII collagen

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IgA plays an important role in immune exclusion at mucosal surfaces and, by binding to its receptor on myeloid cells, triggers a plethora of effector functions. While IgA is important for the immune defence, IgA autoantibodies are associated with various autoimmune conditions. However, a direct pathogenic role of IgA autoantibodies has not yet been demonstrated. Linear IgA disease (LAD), an autoimmune subepidermal blistering disease, is associated with IgA autoantibodies to BP180/type XVII collagen. These autoantibodies preferentially bind to the cleaved 120 kDa ectodomain of this protein. The aim of the present study was to investigate the blister-inducing potential of IgA autoantibodies in LAD. Interestingly, serum from patients with LAD (n=10), but not from normal controls, induced subepidermal splits in cryosections of human skin when co-incubated with leukocytes from healthy volunteers. IgA1 fractions of LAD patients' sera, purified by Jacalin affinity chromatography, retained the blister inducing capacity, while serum depleted of IgA1 was not pathogenic. Recruitment of granulocytes at the dermal-epidermal junction, but not of peripheral blood mononuclear cells, was a prerequisite for split induction. These granulocytes were activated as revealed by their capacity to reduce nitro blue tetrazolium to formazan. $F(ab')_2$ fragments of patients' IgA, lacking the effector Fc portion, lost their split-inducing capacity. Importantly, preadsorption of IgA autoantibodies against the shed ectodomain, but not against a recombinant form of full-length type XVII collagen, abolished their blister-inducing potential. In summary, for the first time, we demonstrate the pathogenicity of autoantibodies of the IgA isotype. In addition, our findings suggest that epitopes, generated by cleavage of the type XVII collagen ectodomain, are important for binding of blister-inducing IgA autoantibodies to this target antigen.

0075

Increased serum calcium and decreased parathyroid hormone levels in patients with palmoplantar pustulosis

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Palmoplantar pustulosis (PPP) is a chronic disease considered to be a variant of psoriasis. Ninety percent of the patients are women and 95% are smokers at the onset of the disease. We have reported that sera from PPP patients give an immunofluorescence (IF) of the papillary endothelium in palmar skin from healthy subjects. In addition, when sections from other organs than skin were screened for IF with PPP sera there was a strong IF in the parathyroid endothelium. This finding raised the question if there might be an abnormal function of the parathyroid gland in conjunction with PPP. Sixty-one women with PPP were compared with regard to the levels of albumin modified serum calcium and parathyroid hormone (PTH) with 155 randomly selected population-based female controls. Age-adjusted mean calcium and PTH levels with 95% confidence intervals (95% CI) were estimated by the GLM procedure of the SAS software. Age-adjusted serum calcium was found to be significantly higher ($p<0.0001$) in patients (mean 2.48 mmol/l; 95% CI 2.46-2.51) compared to controls (mean 2.37 mmol/l; 95% CI 2.35-2.38). Furthermore, we found suppressed PTH levels in cases compared to controls (mean 24.0 nmol/l; 95% CI 20.7-27.3 versus 31.1 nmol/l; 95% CI 29.0-33.2), p for difference 0.0005. In comparison with the control group PPP patients had an odds ratio of 13.0 (95% confidenece interval 4.0-41.8) for diabetes type 2. The results indicate that there is an abnormal calcium homeostasis and parathyroid function in PPP patients resulting in high serum calcium and suppressed PTH levels. It may be speculated that the raised serum calcium is induced by compensatory mechanisms, e g PTH related protein. As mildly elevated calcium levels have been found to be associated with increased prevalence of diabetes the abnormal calcium homeostasis in PPP might be associated with the highly increased risk for diabetes.

0076

Mucous membrane pemphigoid: T- and B-cell reactivity to BP180 predominately targets the C-terminal domain

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Mucous membrane pemphigoid (MMP) and bullous pemphigoid (BP) are distinct subepidermal immunobullous diseases associated with autoantibodies against a common antigen, the transmembrane hemidesmosomal protein BP180. MMP is a heterogeneous disorder and can affect multiple mucosal sites. HLA haplotype associations and activated T-cell infiltrates within lesional histology, provide indirect evidence that cellular immunity has a role in MMP. Our aim was to study T- and B-cell responses against BP180 in MMP relative to BP and normal controls (NC). Eighteen MMP (all with oral involvement), 11 BP and 13 NC subjects were studied. The entire portion of the extracellular domain (ECD) of BP180 was synthesized as three (GST-tagged) overlapping recombinant fragments in E.coli and immunoblotted against the sera. T-cell reactivity to BP180-ECD fusion proteins was assayed by H3-thymidine uptake stimulation studies. Immunoblotting revealed that 61% of MMP (11/18), all BP (11/11), and none of the NC had autoantibodies reactive to BP180 fusion proteins. Within most reactive sera were antibodies to more than 1 portion of the BP180-ECD. Autoantibodies reacted to C-terminal predominantly in MMP (10/18) and to N-terminal in BP (10/11). A similar correlation was seen in T-cell responses, 55% (10/18) of MMP, 54% (6/11) of BP and 23% (3/13) of NC showed T-cell reactivity to BP180. T-cell reactivity to multiple portions of BP180-ECD was seen in both BP and MMP. The specificity of T-cell reactivity did not always predict autoantibody specificity. The incidence of HLA-DQB1*0301 in MMP (75%) was considerably higher than in BP (40%) or NC (23%). Our data suggest that autoreactive T-cells may have a role in the immunopathogenesis of MMP and that specificity of target epitope on BP180-ECD may influence clinical disease phenotype within the pemphigoid group.

0077

Mannose binding lectin in UV-irradiated skin

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Exposure of apoptotic keratinocytes to the immune system is a likely trigger of photosensitive cutaneous autoimmune diseases. We previously reported that dermatomyositis is associated with an increased frequency of three mannose binding lectin (MBL) polymorphisms that have been linked to decreased MBL protein production, MBL54A, MBL57A, and the MBL-LX promoter polymorphism (Werth VP et al. JID in press). We now report the distribution of MBL within normal and UV-irradiated human skin. By immunohistochemistry using a monoclonal anti-MBL antibody (clone 1C10, IgG2b, generous gift of G. Stahl), we detected no MBL in normal skin. 24h after a dose of 30 mJ UVB/cm^2 , MBL was detected on the surface of apoptotic TUNEL-positive keratinocytes in the epidermis. To examine possible sources for this MBL, we irradiated human keratinocytes in vitro then harvested them at 3, 6, and 24 h. Neither MBL mRNA (by RT-PCR) nor MBL protein (by ELISA) were detected, indicating no endogenous MBL production by these cells, even under UVB stimulation. As positive controls, RT-PCR easily detected MBL mRNA in human HuH7 hepatoma cells and TNF mRNA in UV-irradiated keratinocytes. We conclude that non-keratinocyte derived MBL binds to apoptotic keratinocytes after irradiation in vivo, consistent with a possible protective role of MBL in removing apoptotic keratinocytes.

0078

E-cadherin loss by keratinocytes differentiates psoriasiform dermatitis from psoriasis, and cutaneous T cell lymphoma

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Keratinocyte loss of E-cadherin is associated with spongiosis, and results from cleavage of E-cadherin during Fas mediated apoptosis (J Invest Dermatol 117:927-934, 2001). It was hypothesized that loss of keratinocyte E-cadherin may differentiate eczematous psoriasiform dermatitis from psoriasis, and cutaneous T-cell lymphoma (CTCL) / mycosis fungoides, which to not exhibit spongiosis. Fixed, paraffin embedded tissue sections were stained for E-cadherin using monoclonal antibody HECD-1, and immunoperoxidase. Focal loss of E-cadherin was observed in areas of spongiosis in all (9) specimens of spongiotic psoriasiform dermatitis. In contrast, no loss of E-cadherin was observed in the epidermis of (13) slides of psoriasis, or (9) slides of CTCL / mycosis fungoides. This further supports the role of E-cadherin cleavage and Fas mediated apoptosis in spongiotic dermatitis. If confirmed with larger series, E-cadherin may be a useful marker in the differential diagnosis of CTCL.

0079

U-serrated immunodeposition pattern differentiates epidermolysis bullosa acquisita from other subepidermal immunobullous diseases

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Epidermolysis bullosa acquisita can be differentiated from other subepidermal bullous diseases by sophisticated techniques, such as immunoelectron microscopy, salt-split skin antigen mapping, fluorescence overlay antigen mapping, immunoblot and ELISA. We discovered that the diagnosis can also be made by routine direct immunofluorescence microscopy. Studying 156 frozen skin biopsies from patients with various subepidermal immunobullous diseases we found three distinct linear fluorescence patterns at the basement membrane zone: true linear, n-serrated, and u-serrated. The true linear pattern, often seen in conjunction with either the n- or the u-serrated pattern, was found in any subepidermal immunobullous disease with non-granular depositions. In bullous pemphigoid, mucous membrane pemphigoid, anti-epiligrin cicatricial pemphigoid and linear IgA disease the n-serrated pattern was found, corresponding with depositions located in hemidesmosomes, lamina lucida or lamina densa. However, in epidermolysis bullosa acquisita and bullous SLE the u-serrated staining pattern was seen, corresponding with the ultralocalization of type VII collagen in the sublamina densa zone. The diagnosis of epidermolysis bullosa acquisita with IgG or IgA autoantibodies directed against type VII collagen was confirmed by immunoelectron microscopy, salt-split skin antigen mapping, fluorescence overlay antigen mapping, or immunoblot. Using this pattern recognition by direct immunofluorescence microscopy we discovered several cases of epidermolysis bullosa acquisita, who otherwise would have been missed and erroneously diagnosed as a form of pemphigoid or linear IgA disease.

0080

Anti-idiotypic IgG blocks the reactivity and pathogenicity of anti-desmoglein-3 autoantibodies from patients with pemphigus vulgaris

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Idiotypic networks are thought to play an important role in the regulation of immune responses. Applying this strategy, it has recently been demonstrated that specific anti-idiotypic IgG is able to modulate the pathogenic effects induced by autoantibodies in several autoimmune diseases. Pemphigus vulgaris is an autoimmune blistering disease mediated by pathogenic autoantibodies against the desmosomal cadherin, desmoglein-3. In this study, we generated anti-idiotypic IgG against affinity purified Fab fragments of anti-desmoglein-3 autoantibodies isolated from a patient with mucocutaneous pemphigus vulgaris and used it as an interventional tool to counteract the reactivity and pathogenic activity of autoantibodies from other patients with pemphigus. Using ELISAs employing baculovirus-encoded recombinant forms of desmoglein-3 and desmoglein-1, we found that anti-idiotypic IgG was able to inhibit the reactivity of anti-desmoglein-3 autoantibodies from patients with mucocutaneous pemphigus vulgaris in a specific and dose dependent manner. Interestingly, anti-idiotypic IgG did not impair the ability of IgG from patients with pemphigus foliaceus to bind desmoglein-1 in control ELISA experiments. Furthermore, anti-idiotypic IgG neutralized the pathogenic effect of IgG autoantibodies from patients with pemphigus vulgaris in a murine passive transfer animal model. Such inhibition was specific in that anti-idiotypic IgG did not block the ability of autoantibodies from patients with pemphigus foliaceus to elicit superficial, intraepidermal blisters in neonatal mice. These data suggest that pathogenic anti-desmoglein-3 autoantibodies exhibit a common idiotype(s) and that the generation of anti-idiotypic IgG against such idiotype(s) represents a rational approach for blocking the development of this life threatening immunobullous disease.

0081

Endogenous overproduction of TNFα in photosensitive autoimmune diseases

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Exposure of apoptotic keratinocytes to the immune system is a likely trigger of photosensitive cutaneous autoimmune diseases. TNFα, an important mediator of apoptosis, exhibits a promoter polymorphism (–308A) that is overrepresented in SCLE and adult DM and shows increased activity in promoter-reporter constructs in vitro (Werth et al. JID 115:726,2000). In the current study, we sought i) to directly document increased activity of the endogenous TNFα gene in cells derived from SCLE patients with vs. without this genetic polymorphism; and ii) to compare TNFα gene activity in SCLE vs. healthy control cells matched for TNF genotype. Primary skin fibroblasts were grown from SCLE patients with each of the TNF promoter genotypes (AA, GA, and wild-type GG). After UVB irradiation (30 mJ/cm^2), cells from the AA individual showed over 3-fold as much TNFα in culture supernatant relative to the GA and GG cells (207.4 vs. 34.5 and 66.7 ng/ml per 10^6 cells). The SCLE group as a whole produced more TNFα relative to genotype-matched controls (114.8±53.1 pg/ml vs. 8.6±5.6 pg/ml, $p<0.06$, Mann-Whitney test). We next examined TNFα in vivo. In non-diseased control subjects (5/group), basal TNFα staining in skin biopsies showed substantially higher levels in GA (34.5±1.45 AU) than in GG (21.86±1.15 AU, quantitated using Metamorph image analysis, $p<0.001$). We conclude that the –308A polymorphism is associated with increased TNFα accumulation in culture medium and in skin. Importantly, even when matched for TNFα genotype, SCLE cells secrete more TNFα than do cells from healthy controls, suggesting that additional factors may also contribute to TNFα production. Overall, these results are consistent with the model in which increased TNFα secretion accelerates apoptosis, thereby stimulating the immune system leading to autoimmune diseases.

0082

Expression of a dominant-negative AP-1 modulates PKCα and NFκB regulated genes and rescues PKCα overexpressing mice from TPA-induced skin inflammation

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Transgenic mice overexpressing PKCα in the basal layer of epidermis exhibit severe intraepidermal neutrophilic inflammation when treated topically with 12-0-tetradecanoylphorbol-13-acetate (TPA). Keratinocytes from PKCα / 2X TRE-luciferase bigenic mice experienced a sustained increase of AP-1 reporter activity in response to TPA. Crossing PKCα transgenic mice with transgenic mice expressing a dominant negative AP-1 (A-FOS) targeted to skin prevented the TPA-induced inflammatory infiltrate *in vivo*. As detected by confocal microscopy, nuclear factor kappa B (NFκB) translocated to the nucleus of transgenic keratinocytes within 30 minutes of TPA treatment. Among transcripts detected on cDNA microarrays of TPA treated PKCα keratinocytes were several chemokines. As assessed by RT-PCR or RNAse protection assay, expression of transcripts for inflammatory cytokines (TNFα, IL1α) and chemokines (MIP-2, MIP-1α, MIP-3 α) increases in TPA treated PKCα keratinocytes. To elucidate the role of NFκB on TPA-induced gene expression, keratinocytes were infected with the recombinant adenovirus encoding mutated IkappaB super repressor (IκBsr) that locks NFκB in a cytosolic protein complex, preventing its nuclear action. To analyze the role of AP-1, an adenovirus encoding the AP-1 dominant negative (A-FOS) was used similarly. Inhibiting NFκB activation abolished MIP-2, MIP-3α and TNFα induction and decreased the expression of IL-1α and Mip1α transcripts. In contrast, A-FOS further increased the expression of IL-1α and MIP-2 possibly by increasing NFκB binding activity. Together these results implicate PKCα as a regulator of a subset of cutaneous cytokines and chemokines responsible for intraepidermal inflammation. Our results point to a central role of AP-1 transcriptional activity and suggest that PKCα and AP-1 factors could provide novel targets to treat cutaneous inflammation.

0083

Role for Langerhans cell depletion in pathogenesis of psoriasis

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Despite previous reports noting reduced numbers of epidermal Langerhans cells (LCs) in psoriatic plaques (PP skin), neither the mechanism nor the significance of LC depletion is well understood. When superantigen (SA)-activated CD4+ (but not CD8+) T cells are injected into symptomless (PN) skin engrafted onto SCID mice, PP skin is created. However, cellular targets mediating phenotype conversion are unknown. Immunostaining engrafted normal (NN) skin and PN skin revealed presence of epidermal CD1a+/S100+ LCs (n=12). After injection of CD4+ (but not CD8+) T cells, PN grafts converted to PP skin were depleted of epidermal LCs. Also, LCs remained present in NN skin injected with either CD4+ or CD8+ T cells. To determine if SA activated T cells kill LCs, blood-derived CD14+ cells were exposed to GM-CSF/IL-4/TGF-β to produce CD1a+ E-Cad+ LCs, and labeled with ^{51}Cr. Killing of LCs was measured by ^{51}Cr release after 4 hr exposure to T cells at various target:effector ratios (1:5 to 1:40). Comparing mixtures of CD4 plus CD8+ T cells derived from non-psoriatic subjects (at 1:20 ratio) revealed 18 +/-7% killing of LCs (n=9), whereas 48 +/-19% LC killing was observed with psoriatics (n=3). Using negative selection, normal purified SA-activated CD4+ T cells induced killing of LCs (14 +/-5%). When SA-activated mononuclear cells were exposed to either 1,25-$(OH)_2$ Vit D3 or cyclosporin A (n=2), injection into PN skin was accompanied by increased LCs, but no conversion to PP skin. Further evidence linking LC depletion to psoriasis included loss of LCs in PP skin developing in a second animal model using immunodeficient mouse strain-AGR engrafted with PN skin. On day 56, when PP skin spontaneously developed in 5 of 7 grafts, no CD1a+ LCs were present in psoriatic epidermis. Taken together, these results indicate SA-activated CD4+ T cells can kill LCs, and LC depletion may contribute to creation of PP skin. Novel therapies designed to prevent LC depletion and/or replenish epidermal LCs are indicated to restore immunohomeostasis in psoriasis.

0084

Screening for novel targets in severe chronic plaque psoriasis

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The aim of this study was to identify novel target genes for the development of psoriasis treatments. Gene expression was monitored in skin tissue derived from healthy individuals, and compared to lesional and non-lesional skin from psoriasis patients with a PASI score of at least 12. cDNA array based primary screens revealed more than 200 genes to be differentially expressed with a factor greater than 2. Many genes encoded proteins that have been described to be involved in apoptosis or proliferation and formation of the cornified envelope. The regulation of individual transcripts was verified on a single patient level by means of Real time RT-PCR. The spatial expression of a subset of candidate genes was further characterized by in situ hybridization and immunohistochemistry. Selected targets were validated with respect to biological functions crucial in the pathophysiology of the disease, e.g. their modulation affected the proliferation rate of skin cells in vitro. These results demonstrate that our functional genomics approach has yielded valid targets that are suitable for future development of therapeutics in psoriasis.

0085

The clinical and immunological evaluation of pemphigus in the Cukurova region of Turkey

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Pemphigus vulgaris is a potentially life-threatening disease which is more frequently seen in certain ethnic groups and endemic regions. The aim of this study was to determine the clinical and immunological patterns of pemphigus in Cukurova region of Turkey. For this purpose, 50 cases who had administrated to Cukurova University Medical Faculty Department of Dermatology, between November 1998 and April 2002, and diagnosis as pemphigus with clinically, histopathologically and with immunopathologically. The diagnosis were pemphigus vulgaris were 39 patients, pemphigus foliaceus in 11. The age range were 13-85. The male to female ratio was 1:1.3. The mortality rate was 4% (two in pemphigus vulgaris). The association between clinical activation with immunoflourescent and ELISA results were analyzed biostatistically with chi-square test. According to data, a significant relationship betweenclinical activity of the disease and the epidermal and circulating antibody titers was found. However, in spite of this relationship, this assays were not found predictive for relapses. Further studies are needed to determine the genetic and environmental risk factors.

0086

Cutaneous endothelial cell activation in normal skin of patients with dermatitis herpetiformis associated with increased serum levels of IL-8, sE-selectin and sL-selectin

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The mechanisms that lead to skin lesions in DH patients are not known. We have previously demonstrated that circulating neutrophils in patients with DH have an increased expression of CD11b and decreased surface L-sel. Development of an inflammatory response also requires that endothelial cells at the site express adhesion proteins including E-sel, which are controlled by pro-inflammatory cytokines and chemokines including IL-8. We hypothesized that an active immune response in the gut of DH patients would result in elevated levels of IL-8 and be associated with an increased expression of endothelial cell E-sel in the skin and in increased soluble E-sel. Skin biopsies were obtained from the inner arm of 7 DH patients and 11 normal subjects, RNA isolated, and cDNA prepared. Real-time PCR was performed to measure the expression of E-sel and ICAM-1. Sera from 51 patients with DH were analyzed for the presence of IL-8, sE-sel and sL-sel. DH patients' skin expressed markedly increased levels of E-sel mRNA. Mean E-sel mRNA expression in DH skin was 26.8 (range 5.5 -54.4) times greater than that of a control, normal skin($p<0.001$). No increased expression of ICAM-1 was seen in DH skin. Increased serum IL-8 was seen in 41% of DH patients(range 3.2 - 5,900 pg/ml; nl < 31.2 pg/ml). Elevated levels of sE-sel (37%) and sL-sel(35%)were seen in some DH patients. Serum IL-8 levels decreased in 2 of 2 patients after being on a GFD and sE-sel and sL-sel decreased in 1 of 2 patients. These studies demonstrate that patients with DH have elevated levels of serum IL-8, increased expression of E-sel in the skin and that this may be a result of the gut inflammatory response to gluten. Endothelial cell activation along with neutrophil priming may play a critical role in the development of skin lesions in patients with DH and may represent a common mechanism for other cutaneous manifestations of inflammatory disease of the gut.

0087

Autocrine regulation of tumor necrosis factor-α in keratinocytes

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TNFα is critically implicated in the regulation of many inflammatory diseases, including Psoriasis and Contact Dermatitis. However, the induction and regulation of TNFα in skin not fully described. In this study, we investigated whether autocrine regulation of TNFα is present in the skin. Using the BALB/c-derived PAM212 and the C3H-derived HDII keratinocyte cell lines, we performed in vitro stimulations using recombinant murine TNFα. We observed a dose-response of TNFα-mediated induction of TNFα in keratinocytes using both cell lines. To study the intracellular signalling mechanisms involved in the observed increased expression of TNFα mRNA, we addressed the importance of different protein kinases. Using selective inhibitors, we found that blocking PKC- but not PKA-dependent signalling mechanisms, a dose-dependent inhibition of the autocrine regulation of TNFα in keratinocytes was found. To determine whether the increase in TNFα mRNA observed upon stimulation with rTNFα was due to an increase in promoter activity of the TNFα gene and thus represented an increase in transcription, HDII cells were transfected with a Chloramphenicol acethyl transferase (CAT) construct containing the full length (minus 1059bp to +138bp) TNFα promoter fragment, before stimulations. This resulted in an increase in transcription of the TNFα gene in the keratinocytes. Furthermore, no change in stability of the TNFα mRNA was detectable thus excluding significant translational regulation. Thus, an autocrine regulation of TNFα in keratinocytes was demonstrated. The induction was regulated at the transcriptional level and required PKC-dependant signalling. These results indicate that even a minor induction of pro-inflammatory cytokines in skin could have profound effects. Finally the observation of autocrine regulatory pathways in keratinocytes implicates the keratinocyte as an amplifier with regard to pro-inflammatory cytokine secretion

0088

An animal model of atopic dermatitis: cDNA microarray analyses of mRNA in chronic lesional skin delineate a balanced Th1/Th2 inflammatory cytokine profile

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An animal model of chronic inflammatory skin disease has been developed in an epidermally expressed IL-4 Transgenic (IL-4-Tg) mouse line closely resembling human atopic dermatitis (AD) [J Invest Dermatol 2001; 117:977-83]. To define the in-situ inflammatory cytokines and mediators, cDNA microarray analyses on 96 gene-coded membranes were performed with reversely transcribed mRNA extracted from chronic lesional (>30 disease days) and non-lesional (NL) skin of 7 PCR-confirmed IL-4-Tg mice and skin of 2 age-matched Non-Tg mice. DNA hybridization followed by chemiluminescence autoradiography was used to determine the cytokine and mediator levels, normalized to house-keeping gene GAPDH. Most of the cytokine mRNAs examined were highly increased in the lesional skin when compared to the NL and non-Tg mouse skin respectively, with the average fold increase depicted below: IL-4, 2.0, 6.8; IL-3, 7.6, 9.9; IL-5, 5.6, 9.7; IL-10, 4.5, 3.9; IL-13, 2.1, 4.1; IL-2, 2.4, 3.2; IL-12p40, 7.4, 5.4; IFN-γ, 3.4, 8.8; IL-1β, 11.1, 23.1; IL-6, 4.9, 11.0; IL-17, 7.7, 13.4; TNF-α, 1.5, 4.9; TNF-β, 4.5, 5.7; G-CSF, 4, 6.6; GM-CSF, 2.9, 4.8. Co-stimulation molecules were similarly increased in folds: CD40L, 2.5, 4.4; OX40L, 1.7, 4.7. In addition, these molecules were also increased many folds in the NL skin when compared to non-Tg mouse skin: IL-4, 3.6; IL-3, 1.5; IL-5, 4.3; IL-10, 2.2; IL-13, 3.5; IL-2, 1.9; IL-12p40, 2.8; IFN-γ, 6.9; IL-1β, 2.6; IL-6, 4.3; IL-17, 2.0; TNF-α, 4.4; TNF-β, 3.0; G-CSF, 5.6; GM-CSF, 3.8; CD40L, 2.4; OX40L, 2.9. The ratio of fold-increase in lesional skin compared to non-Tg mice for the critical Th2:Th1 cytokines were close to one: IL-4:IL-12p40, 1.3; IL-4:IFN-γ, 0.8. The highest fold of increase was in IL-1β, a non-Th-specific inflammatory cytokine. Together, our data delineate a balanced Th1/Th2 cytokine profile in chronic lesional skin, thus further supports a close resemblance of our IL-4-Tg mouse disease model with human AD.

0089

Humoral and cellular immune responses against human papillomavirus type 5 proteins favor its involvement in the immunopathogenesis of psoriasis

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In our previous studies we found in 90% of psoriatic plaques DNA of epidermodysplasia verruciformis (EV) HPVs, mainly oncogenic HPV5, as well as in over 25% of the patients presence of antibodies against HPV5L1 capsid epitopes. To find out whether EV HPVs are involved in the immunopathogenesis of psoriasis we studied prevalence of specific antibodies against HPV5 E6 and E7 oncoproteins by means of radio-immunoprecipitation assay with in vitro translated E6/E7 proteins. Cell-mediated immunity (CMI) was studied by the T cell proliferation assay with the use of HPV5a VLPs (virus-like particles). Production of cytokines by VLP-stimulated PBMC was measured by specific ELISA. Antibodies against E6/E7 HPV5 proteins were found in a very high percentage (51.7%) of psoriatic patients (n=29), comparable to that (55.6%) in EV patients (n=9), and were rarely (3.1%) detected in atopic dermatitis (n=32) and in healthy individuals (2.3%, n=42). The presence of anti-E6/E7 antibodies provides strong evidence that in the psoriatic epidermis EV HPV5 life cycle is activated and the viral oncoproteins known to stimulate keratinocyte proliferation are expressed. Studies on CMI showed that HPV5 VLPs stimulated T lymphocyte proliferation in patients with psoriasis (n=7), and this was associated with an increased production of IFNg ($p<0.05$), cytokine crucially involved in the pathogenesis of psoriasis. In contrast, T lymphocytes from EV patients (n=4) did not respond to HPV5a VLPs, as measured by the proliferation assay and IFNg production. Our data support the hypothesis that HPV5 and other EV-HPVs are involved in the pathogenesis of psoriasis both by direct stimulation of keratinocyte proliferation (by E6/E7 oncoproteins) and, indirectly, by serving as a target for humoral and cellular (auto)immune reactions.

0090

Transfer of alopecia areata in the human scalp graft/prkdc^{scid} (SCID) mouse system is characterized by a TH1 response

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Alopecia areata is an autoimmune condition directed at hair follicles, which results in loss of hair. It is possible to transfer hair loss, along with the immunohistologic findings of alopecia areata, to human scalp grafts on Prkdc^{scid} (SCID), mice by injection of autologous activated lesional T-cells. This study examines the cytokine profile of T-cells and follicular epithelium following transfer of hair loss. Two consistent findings significantly associated ($p<0.01$) with hair loss were production of interferon-gamma inducible protein-10kD (IP-10) by follicular epithelium (13/13), and production of interferon-gamma (INF-gamma) by infiltrating T-cells (10/12). Non-injected control grafts re-grew hair, and were generally negative for IP-10 (positive 2/9), and INF-gamma (positive 2/9), but expressed of IL-10 on the follicular epithelium (7/9). IL-4 and TNF-alpha were each expressed by infiltrating cells in only (1/12) grafts with hair loss, suggesting that TNF-alpha inhibitors may not be clinically useful. This data supports an INF-gamma TH1 pathogenesis for hair loss in alopecia areata. Biological response modifiers directed at INF-gamma and TH1 T-cell responses may be useful in the therapy of alopecia areata.

0091

Exaggerated B cell signaling by disrupted CD22/SHP-1 inhibitory regulation in the tight-skin mouse

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Positive and negative signals from extracellular stimuli control lymphocyte responses and maintain self-tolerance, and altered balance of signal transduction in lymphocytes may result in development of autoimmune diseases. The tight-skin (TSK) mouse, a genetic animal model for human systemic sclerosis (SSc), develops cutaneous fibrosis resembling SSc. While the known TSK mutation is in the gene encoding the extracellular matrix glycoprotein fibrillin 1, TSK mice exhibit a skewed humoral response and produce autoantibodies specific and characteristic for human SSc. We assessed the molecular mechanisms that established abnormal B cell signaling thresholds in TSK mice. Remarkably, circulating B cells from TSK mice exhibited characteristic phenotype of chronically activated B cells with down-regulated surface IgM expression and up-regulated MHC class II expression, suggesting that TSK B cells had intrinsic abnormalities in B cell antigen receptor (BCR) signal transduction. Analyses of intracellular signaling events revealed that BCR cross-linking induced exaggerated calcium responses and augmented activation of ERK activation in TSK B cells. Furthermore, functions of CD22, a B cell-specific siglec protein, was specifically impaired in TSK B cells since BCR ligation induced significantly diminished CD22 phosphorylation as well as modest SHP-1 association with CD22 in TSK B cells. Consistently, CD19, a major target of CD22 negative regulation, was hyperphosphorylated in TSK B cells after BCR ligation. Additionally, analyses of CD22-deficient TSK B cells demonstrated that hyperactivation of TSK B cells was dependent on CD22 expression. As have been recently reported, signal termination provided by negative regulatory pathways such as Lyn/CD22/SHP-1 complex play a key role in preventing autoimmune reactions. These findings suggest that disrupted inhibitory signal provided by CD22 contributes to the characteristic features of autoimmunity in TSK mice and provide a novel potential therapeutic target of SSc.

0092

Disruption of tumor necrosis factor receptor p55 accelerates collagens deposition in an animal model of scleroderma

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Increases in the serum concentration of the soluble tumor necrosis factor receptor p55 (TNFRp55) correlate with scleroderma severity and inhibit TNF- mediated signaling. However, there is no direct evidence for TNFRp55 mediating the specific functions of TNF in the disease. To determine the role of TNFRp55-mediated signaling in the pathogenesis of scleroderma, wild-type and TNFRp55 deficient (TNFRp55-/-) mice received a subcutaneous injection of bleomycin (BLM) each day. TNFRp55-/- mice began to develop severe sclerotic changes of the dermis on day 3 of the subcutaneous injections of BLM, and produced anti-nuclear antibodies, while wild-type mice did not. The collagen content in the BLM-treated TNFRp55-/- skin after 5 days of injections was almost 3-fold that in the skin of the untreated mice and the BLM-treated wild-type mice. The expression levels of fibrogenic cytokines, procollagen alpha 1 and the matrixmetalloproteinase (MMP)-9 mRNAs were unaffected in both wild-type and TNFRp55-/- skin, with or without BLM treatment. Induction of MMP-1 protein expression was significantly inhibited, and induction of MMP-2 expression maintained longer in the BLM-treated TNFR p55-/- skin. These results raised the hypothesis that aberrant expression of MMP-1, and -2 expression in TNFRp55-/- mice might cause severe accumulation of collagen in BLM-treated skin. On the one hand, BLM-treated wild-type mice exhibited sclerosis of skin after about 3 weeks of BLM-injection, whereas saline-treated group did not. Interestingly, significant elevation of sTNFRp55 in sera was observed in BLM-treated wild-type mice. This reproducibility of this phenotype among these BLM-induced mice models demonstrated that impaired TNFRp55-signaling might also occur in scleroderma patients. This report might provide a basis for understanding the pathogenesis of scleroderma and formulating therapeutic intervention.

0093

Protein expression profile of acute guttate psoriasis lesions

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Protein patterns in lesional and non-lesional skin of 11 patients with acute, untreated guttate psoriasis triggered by streptococcal throat infection were analysed using two-dimensional polyacrylamide gel electrophoresis (2-DE) and compared with normal healthy skin from controls and acute induced contact eczema. The biopsies were taken from the same body location in all subjects. Protein expression pattern was highly reproducible between samples. The expression pattern was evaluated by computer-assisted image analysis (PDQUEST). Forty-five proteins had a statistically significant differential expression between lesional and non-lesional psoriatic skin. These proteins were used in hierarchal cluster analysis of control, non-lesional and lesional psoriasis skin. All psoriasis non-lesional skin samples clustered with the control skin and all psoriasis lesions were distinctly clustered. When the same analysis was done for control skin and psoriatic skin lesions, expression of 47 protein spots differed significantly. Examining the overlap between the two datasets revealed 13 spots common to both sets. The 45 protein spot dataset was re-evaluated after adding data from eczematous and healthy skin samples. The resulting dataset of 41 proteins that remained differentially expressed could be used to classify the samples correctly into three groups with control skin and non-lesional psoriatic skin in one group and lesional psoriatic and eczematous skin in the other two groups. Protein expression data also revealed a distinct set of 13 proteins that showed at least twofold up-or down regulation between psoriatic lesions and normal skin. These proteins were identified using matrix assisted laser desorption/ionization mass spectrometry. This study demonstrates the potential to discover psoriasis specific proteins using proteome analysis and the potential for objective classification of such lesions.

0094

Bullous pemphigold IgE and IgG react with BP180 site outside the immunodominant NC16A domain

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Bullous pemphigoid (BP) is an autoimmune skin disease characterized by the development of autoantibodies directed against the hemidesmosomal protein BP180 (BPAg2, type XVII collagen). IgG from the majority of BP sera reacts within the 75 amino acid non-collagenous domain 16 of BP180, termed NC16A. In addition to IgG antibodies, IgE class antibodies directed against BP180 are also present in the sera of BP patients. We have recently shown that mast cells in the skin of BP patients are coated with IgE and fragments of BP180, and the circulating basophils from these patients undergo antigen-specific degranulation when exposed to recombinant BP180. The current studies were designed to identify in more detail the regions of BP180 with which the BP180-specific IgE reacts. 30 BP patients were initially studied by ELISA and immunoblotting. Twenty-two (73%) had IgG reactivity to NC16A by ELISA and 15 (50%) had IgE reactivity to NC16A. One patient was identified that had IgG and IgE reactivity to full-length BP180 extracted from keratinocytes but no reactivity to NC16A, suggesting he had reactivity exclusively outside the NC16A domain. Seven patients were studied in more detail. In order to facilitate these studies two constructs were used: one encompassing the entire extracellular domain of BP180 termed sec180 and sec180ΔNC16A, the extracellular domain from which the NC16A region had been deleted. BP patients were tested for IgG and IgE reactivity with these two recombinant proteins by immunoblotting. 4/7 were positive for IgG reactive with sec180 and 2/7 reacted with sec180ΔNC16A. IgE reactivity in these 7 patients paralleled IgG reactivity. One patient was identified with IgG and IgE reactivity exclusively outside the NC16A domain. This suggests that in addition to NC16A, epitopes outside this region may be pathogenically relevant.

0095

Normal wound healing in mice lacking the platelet-activating factor receptor

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Platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine; PAF) is a glycerophosphocholine-derived lipid mediator implicated in numerous inflammatory processes. PAF is synthesized by cells in response to numerous stimuli, including cytokines, growth factors, and cellular injury. PAF acts via a specific receptor (PAF-R), a G-protein coupled transmembrane protein. Many cells important in wound healing including neutrophils, moncocyte/macrophages, endothelial cells and keratinocytes all synthesize this mediator and express PAF-Rs. The objective of this study was to assess the role of the PAF signaling system in a murine model of wound healing. Wild-type C57BL6 or mice deficient in PAF-Rs (J. Exp. Med. 187:1779-1788, 1998) were subjected to a 5 mm punch biopsy on back skin, and the rate of wound healing was monitored over time. The healing rate of PAF-R-negative and normal mice appeared equal, with all wounds completely healed by 14-18 days. These studies suggest that the PAF-R is not essential in this murine model of wound healing.

0096

Systemic exposure to the inflammatory lipid mediator platelet-activating factor results in immunosuppression in mice

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Platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine; PAF) is a glycerophosphocholine-derived lipid mediator implicated in numerous inflammatory processes. Epithelial cells synthesize PAF and related 1-acyl-PAF-like species in response to various stimuli including ultraviolet light irradiation (UVB), ionophores, or cellular damage. In addition to producing PAF, cells ranging from granulocytes to keratinocytes also express the PAF receptor (PAF-R). Activation of the epidermal PAF-R leads to the production and release of PAF, IL-6, IL-8, IL-10, TNF, and eicosanoids. Given that activation of the PAF system induces both pro- and anti-inflammatory cytokines, the objective of these studies was to define the effects of a systemic PAF-R agonist on immune function in a delayed-type-hypersensitivity model. C57BL6 Mice were sensitized with the topical antigen dinitrofluorobenzene (DNFB) on shaved back skin. Seven days after sensitization, the ears of mice were painted with either DNFB or vehicle control. Twenty-four hours after application of DNFB to ears, 5 mm punch biopsies were obtained and biopsy thickness and weight were assessed as measures of the inflammatory response. To examine the role of PAF, mice were systemically pre-treated with various doses of the PAF-R agonist carbamoyl-PAF (CPAF) through intraperitoneal administration, prior to sensitization with DNFB. In alternate experiments, mice were systemically exposed to the known immunosuppressant UVB radiation prior to sensitization with DNFB. Intraperitoneal injection of CPAF resulted in a significant inhibition of allergic contact dermatitis in this model system. Furthermore the inhibition of immune response induced by CPAF was comparable to the inhibition observed following ultraviolet B radiation. These studies suggest that systemic exposure to a PAF agonist results in an immunosuppressive response and could indicate a putative role of PAF in UVB-induced immunosuppresion.

0097

Augmentation of chemotherapeutic agent-induced cytokine production by expression of the platelet-activating factor receptor in a human epithelial carcinoma cell line

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In addition to their known cytotoxic effects, chemotherapeutic agents can also trigger cytokine production in tumor cells. Many chemotherapeutic agents are also potent pro-oxidative stressors. Although the lipid mediator platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine; PAF) is synthesized in response to oxidative stress, and many epidermal carcinomas express PAF receptors (PAF-R) linked to cytokine production, it is not known whether PAF is involved in chemotherapeutic agent-induced cytokine production. These studies examined the role of the PAF system in chemotherapy-mediated cytokine production using a model system created by retroviral-mediated transduction of the PAF receptor-negative human epidermal carcinoma cell line KB with the human PAF-R. The presence of the PAF-R in KB cells resulted in an augmentation of production of cytokines interleukin-8 and tumor necrosis factor-alpha induced by chemotherapeutic agents etoposide and mitomycin C. Oxidative stress was found to mediate chemotherapeutic agent-induced activation of the epidermal PAF-R because it was mimicked by treatment with an organic hydroperoxide, and inhibited by antioxidants. The transcription factor nuclear factor kappa (NFkappa)-B was also essential to this cytokine production augmentative effect, because it was blocked by inhibition of the NFkappaB pathway using a super-repressor IkappaBM mutant. These studies provide evidence for a novel pathway whereby the epidermal PAF-R can augment chemotherapy-induced cytokine production through an NFkappaB-dependent process.

0098

Evidence for a pro-inflammatory role of proteinase-activated receptor-2 during cutaneous inflammation *in vivo*

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Contact dermatitis (CD) is a frequent dermatological disease characterized by acute to chronic inflammation of the skin. Proteinase-activated receptor-2 (PAR2), a G protein-coupled receptor for certain proteases, is localized on keratinocytes, endothelial cells and nerve fibers, and has been shown to play a role during inflammation of several tissues. However, the underlying mechanism of PAR2-induced regulation of inflammation is still fragmentary. Therefore, we were interested in whether or not PAR2 is involved in cutaneous inflammation using a model of induceable allergic (ACD) and irritant (ICD) contact dermatitis. In wild-type (PAR2+/+) mice, PAR2 agonists induced an increased intradermal edema and enhanced plasma extravasation. These inflammatory responses were significantly diminished in PAR2-deficient (PAR2-/-) mice and controls. Morphological analysis revealed a dramatic increase of spongiosis and intradermal edema along with enhanced infiltration of neutrophils and monocytes in PAR2+/+ mice as compared to PAR2-/- mice. Interestingly, nitric oxide (NO) inhibitors significantly diminished these effects indicating a role of NO in PAR2-induced inflammatory responses of the skin. Functional studies at the RNA- and protein level further revealed PAR2-induced upregulation of the cell adhesion molecules ICAM-1 and E-selectin by HDMEC during inflammation suggesting that PAR2 directly regulates CAM function during skin inflammation. PAR2 agonists also stimulated upregulation of mediators involved in cutaneous inflammatory responses such as IL-6 and NO in murine and human (dermal) endothelial cells. Together, these results strongly suggest a proinflammatory role of PAR2 during CD and probably other inflammatory dermatoses, characterized by edema, plasma extravasation and recruitment of inflammatory cells. Thus, PAR2 antagonists may be therapeutic tools for the treatment of inflammatory skin disorders such as CD and atopic eczema.

0099

Regulatory T cells are induced by enhanced RANK-RANKL signaling in transgenic mice

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RANK-RANKL interaction plays an important role in the regulation of T cell/DC communication, DC survival as well as lymph node formation. RANK is expressed on DC, whereas RANKL is found on activated T cells. Accordingly, epidermal Langerhans cells (LC) express RANK and RANKL transcripts are detectable in skin upon inflammation. To investigate the role of RANK-RANKL signaling in cutaneous immunity, transgenic (tg) mice were generated that overexpress RANKL under control of the skin-specific Keratin-14 (K14) promoter. K14-RANKL-tg mice develop normally and have a uniform expression of the transgene in basal keratinocytes yet RANKL is not detectable in the serum. The number of epidermal LC is normal in tg mice. Blockade of RANK-RANKL interactions has been described to inhibit the development of CD4+CD25+ regulatory T cells (Treg). Since in K14-RANKL-tg mice increased signaling via this pathway is induced, we hypothesized that these tg mice harbor enhanced numbers of Treg. Indeed, a 2-3-fold increase in numbers of CD4+CD25+ Treg cells is found in K14-RANKL-tg mice. These Treg cells are characterized by expression of GITR, CTLA-4, and CD45RBlow. In vitro, the Treg cells from tg mice were anergic, suppressive and produced IL-10. The anergic state could be overcome by stimulation with CD3, CD28 and IL-2. To investigate if these Treg cells from tg mice were functional in vivo contact hypersensitivity (CHS) was induced using DNFB as a hapten. Tg mice show significantly reduced CHS responses. This suppression was transferable since injection of isolated Treg cells from DNFB-sensitized K14-RANKL-tg mice into littermates previously immunized with DNFB inhibited the elicitation of CHS. This suppression was antigen-specific because transfer into oxazolone-immunized recipients failed to inhibit the elicitation of CHS. Treg cells play an integral role in controlling autoimmunity. Therefore, we used K14-CD40L-tg mice, which develop severe systemic autoimmune disease, including auto reactive T cells and autoantibody, and crossed them to K14-RANKL-tg mice. The double transgenic did not develop autoimmune disease implying that the RANK-RANKL induced Treg suppressed the K14-CD40L-induced autoimmune phenotype. Together, these data provide first evidence that stimulation of RANK-RANKL signaling by overexpression of RANKL induces Treg cells in vivo.

0100

Pemphigus vulgaris IgG-induced acantholysis in organ and cell culture—kinetics of appearance of apoptotic effectors

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We have shown before that acantholytic lesions in *pemphigus vulgaris* (PV) patients' skin show abundance of apoptotic cells that are enriched with apoptotic proteins like P53, Bax etc. Furthermore we have shown that PV-IgG in cell and organ culture (in the presence of interferon-γ) causes acantholytic lesions. Lesional cells are enriched in apoptotic markers like FasR, FasL, caspases and reveal degraded DNA, characteristic of apoptosis. In order to address the question if apoptosis induces acantholysis or *vice versa*, we followed the appearance of apoptotic markers in cell and organ cultures treated by PV-IgG in respect to acantholytic lesion formation. Our data show up-regulation of apoptotic effectors at early time points (10-30 h), when no apparent change in cell-cell contacts was observed, while acantholytic lesions appeared only after 2-3 days of incubation with PV-IgG. Normal human keratinocytes treated with PV-IgG displayed time-dependent secretion of soluble (onset at 5 h incubation) and membranal FasL, FasR, enrichment in caspase 8 and activation of caspases 1 and 3. In skin organ culture, immunohistochemistry revealed mobilization/membranal localization of DISC members, FADD and caspase 8, preceding formation of the intraepidermal blister by ~ 24 h. The typical suprabasilar split in organ culture appeared after prolonged incubation (52 h) and correlated very well with the further elevation of caspase 3 activity. Our data provide the first insight into the molecular mechanism of PV-IgG-induced acantholysis, and highlight a possibility of apoptosis induction as a plausible mechanism in induction of acantholysis by the *pemphigus* autoimmune antibodies.

0101

Differential sets of immunodominant T cell epitopes of desmoglein 3 are recognized by autoreactive T cells from pemphigus vulgaris patients and healthy individuals

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Pemphigus vulgaris (PV) is an autoimmune mediated disease, associated with circulating auto-Ab to the desmosomal glycoprotein desmoglein 3 (Dsg3). Previous studies from our lab and others identified autoreactive Th1 and Th2 cells against Dsg3 supporting the idea, that autoreactive T cells are critical for the induction and regulation of Ab production in PV. Moreover, healthy carriers of the PV-prevalent HLA class II alleles, HLA-DRB1*0402 and HLA-DQB1*0503, showed an exclusive Th1 cell response against Dsg3 at frequencies similar to those in patients, indicating that distinct HLA class II alleles seem to be important for T cell recognition of Dsg3 peptides. The aim of this study was to characterize T cell recognition of Dsg3 in patients and Dsg3-responsive healthy individuals. Dsg3-reactive Th1 and Th2 clones (isolated by MACS secretion assay) from PV patients (n=14) and healthy donors (n=13) were co-cultured with 17mer peptides of the extracellular domain (ECD) 1-5 of Dsg3 and HLA class II matched APC. Proliferative response was determined by the incorporation of 3H-thymidine. Both, Dsg3-autoreactive Th1 and Th2 cells from PV patients recognized a limited set of epitopes located in the ECD1 (DG2), ECD2 (DG3, DG4, DG5) and ECD3 (DG6, DG7). Dsg3-reactive T cells from healthy donors showed responsiveness to Dsg3-peptides DG2, DG5 and DG8 located in the ECD1, ECD2 and ECD5, respectively. All the identified Dsg3 peptides exhibited similar or identical residues at positions 1 and 4 and some of them also at position 6, representing most likely anchor motives with the restricting HLA class II alleles. Our findings strongly suggest that T cell recognition of Dsg3 is restricted to limited epitopes of the ECD of Dsg3 and thus differential epitope recognition of Dsg3 in patients and healthy donors may contribute to the different clinical phenotypes.

0102

Psoriasin expression in normal and diseased skin

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Psoriasin is a member of the S100 gene family and was first detected in abnormally proliferating keratinocytes in psoriatic epidermis. Subsequently, it was found to be up-regulated in abnormally differentiating keratinocytes in addition to breast and bladder cancer. Psoriasin may exert an effect as a chemotactic factor for inflammatory cells. Very recently psoriasin has been shown to display antimicrobial activity against E. coli by depletion of zinc ions. The purpose of this study was to determine the patterns of psoriasin expression in infectious and noninfectious inflammatory skin disorders. The expression of psoriasin mRNA and protein was determined by in situ hybridisation using a specific probe generated in the lab and immunohistochemically using a commercially available monoclonal antibody on paraffin sections of normal human skin of different localisations and in several inflammatory skin disorders. Expression of psoriasin mRNA was constitutively high in epidermis of normal skin whereas immunoreactivity was found predominantly in areas which are highly exposed to both, microbial challenge and UV-radiation (e.g. nose, forehead). In inflammatory skin diseases, the mRNA expression of psoriasin in the epidermis was generally high and increased immunoreactivity was found in psoriasis, folliculitis and condyloma acuminata throughout the spinous layer whereas patients with lichen ruber or eczema showed only little staining in upper parts of the epidermis. This study demonstrates the modulation of psoriasin expression in both healthy skin and infectious/ non-infectious skin diseases. The distinct expression in psoriatic skin explains the clinical experience of a low incidence of bacterial infection as well as indicating biological functions of the protein yet unknown, but beyond antibacterial activity.

0103

CD44-Hyaluronan interactions mediate lymphocyte adherence to dermal endothelium under shear stress in acute cutaneous graft-versus-host disease

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Acute graft-versus-host disease (aGVHD) is a major complication of allogeneic hematopoietic stem cell transplantation (HSCT), which is a treatment of choice for certain hematologic malignancies and genetic disorders. Acute cutaneous GVHD (acGVHD) is the most frequent presentation of this disease. Lymphocytic infiltrates of the papillary dermis are the earliest feature of acGVHD and occur at times of profound lymphopenia, indicating that the migration of lymphocytes from vascular to dermal compartments is highly efficient. To examine the molecular basis of this lymphocytotropism, we performed adherence assays to analyze lymphocyte-dermal endothelial interactions under shear stress conditions which mimic blood flow. We observed binding of lymphocytes to dermal endothelial cells in acGVHD reactions, but not in other post-HSCT skin eruptions. Pre-incubation of lymphocytes with functional blocking antibodies against LFA-1 and L-selectin, as well as treatment of skin sections with blocking antibodies directed against the endothelial proteins E-selectin, P-selectin, ICAM-1 and VCAM-1, had no effect on lymphocyte adherence to dermal endothelium. Neuraminidase and keratanase digestions of skin sections had no effect on binding of lymphocytes to endothelium, whereas heparitinase and chondroitinase ABC digestion partially blocked, and hyaluronidase digestion completely blocked binding. Consistent with these findings, lymphocyte adherence to dermal vessels under shear was abrogated by pre-incubation of lymphocytes with function blocking anti-CD44 monoclonal antibody (Hermes 1) or by soluble hyaluronan (HA). Immunohistochemical staining for HA showed discrete HA depositions at endothelium supporting lymphocyte binding. Taken together, our data suggest that recruitment of lymphocytes to skin in acGVHD depends in part on upregulated endothelial expression of HA and that analysis of HA expression on dermal endothelium may aid in diagnosis and management of acGVHD.

0104

Detection of RNase 7 immunoreactivity in inflammatory skin diseases

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The skin as the largest human organ is constantly exposed to various microbes. Surprisingly however the incidence of clinically manifest skin infections is rather low. Apparently, there are potent protective mechanisms which counteract microbial attacks. A 14.5-kDa antimicrobial ribonuclease, termed RNase 7, was isolated from skin-derived stratum corneum. RNase 7 exhibited potent ribonuclease activity and revealed broad spectrum antimicrobial activity against many pathogenic microorganisms and remarkably potent activity (lethal dose of 90% < 30 nM) against a vancomycin-resistant Enterococcus faecium. To obtain antibodies against Rnase, 7 Balb/c mice were immunized with natural protein purified from psoriatic scales. One out of several monoclonal antibodies raised showed distinct epidermal immunoreactivity on both acetone fixed cryostat sections and formaldehyde fixed, paraffin embedded sections of human skin. In normal human skin, RNase 7 immunoreactivity was found in all epidermal layers and remarkably high in biopsies of the trunk. In chronic plaque type psoriasis, signals were detected in upper layers below and within parakeratotic layers around microabscesses. Eczema showed a similar pattern whereas in folliculitis a diffuse epidermal staining was found. Surprisingly, dermal vasculature is stained as well. These findings support the idea of an important role of antimicrobial peptides in skin disorders beside protection from infections.

0105

Critical role of galectin-3 in phagocytosis by macrophages

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Macrophages are functionally important in the skin as scavengers of apoptotic cells and invading pathogens through their phagocytic activity. However, signal transduction into macrophages following extracellular stimuli for phagocytosis is not fully understood. As a member of a large family of animal lectins, galectin-3 is also expressed abundantly in macrophages, but its functions in this cell type are not clearly defined. We have studied the effect of galectin-3 gene targeting on the phagocytic function of macrophages. Compared to wild-type macrophages, galectin-3-deficient (gal3-/-) cells exhibited reduced phagocytosis of IgG-opsonized erythrocytes and apoptotic thymocytes in vitro. Gal3-/- mice showed attenuated phagocytic clearance of apoptotic thymocytes by peritoneal macrophages in vivo, and reduced IgG-mediated phagocytosis of erythrocytes by Kupffer cells, in a murine model of autoimmune hemolytic anemia. Gal3-/- macrophages exhibited a lower degree of actin rearrangement upon FcR crosslinkage. Additional experiments indicate that extracellular galectin-3 does not contribute appreciably to the phagocytosis-promoting function of this protein. Moreover, confocal microscopic analysis of macrophages containing phagocytosed erythrocytes revealed localization of galectin-3 in phagocytic cups and phagosomes. Finally, gal3-/- macrophages showed significantly reduced activation of protein kinases Lyn and Syk upon FcR cross-linkage. These results indicate that galectin-3 contributes to macrophage phagocytosis through an intracellular mechanism by regulating critical signaling pathways. Thus, galectin-3 may play an important role in both innate and adaptive immunity by contributing to phagocytic clearance of microorganisms and apoptotic cells.

0106

Cross-reactivity among candidate autoantigens in psoriasis

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The etiology of psoriasis is unknown, but is thought to be the result of an autoimmune reaction against a skin antigen or antigens. We have undertaken an expression cloning project to identify these autoantigens, and have previously reported three candidate proteins. These are keratin 13, a mucocutaneous keratin upregulated in wound healing; hnRNP-A1, a spliceosome component previously implicated in rheumatoid arthritis; and a previously uncharacterized protein which we refer to as PAAg-1. We now report results of detailed screening of serum samples to all three of these antigens. As is the case with autoantigens in other autoimmune diseases, reactivity was found in subsets of both patients and healthy controls. However, we also made the surprising observation that subjects which react to one protein often react to two or all three proteins. Two potential explanations are that these subjects have a higher degree of general autoreactivity, or that the antibodies cross-react. To test these explanations, we immunoprecipitated serum antibodies to keratin 13, and found that these antibodies do in fact cross-react with hnRNP-A1 and PAAg-1. We draw two conclusions from this: (1) the epitope we have identified does appear to be immunodominant, strengthening the possibility that it is of significance in psoriasis, and (2) the cross-reactivity between a skin keratin and an antigen previously implicated in arthritis may be related to the association between skin psoriasis and psoriatic arthritis. Finally, we present evidence that an additional cross-reactivity of these antibodies is to the tax protein of HTLV-1. Although typical HTLV-1 infection is not seen in psoriasis patients, a relationship between psoriasis and a "tax-only" carrier state or exposure to a related retrovirus are intriguing possibilities.

0107

Non-invasive sampling and cytokine profiling for prediction of irritant skin reactions

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This study examined the ability of a non-invasive tape harvesting technique, coupled with cytokine mRNA profiling to: 1] predict the ability of a dilute irritant (0.05% SLS) to cause irritation after extended occlusive patching; and 2] predict the future severity of a skin reaction after 6 hours of occlusive patching of an irritant (1.0% SLS). In two different trials, up to 10 subjects were patched with SLS and water from 6 hours to 9 days with differing concentrations of SLS. The sites were then shave biopsied or harvested using Epidermal Genetic Information Retrieval adhesive films (EGIR films). The films are used to collects skin cells from the surface of the skin. With the EGIR technique, 4 adhesive tapes are applied to a skin site and removed. The cells adherent to the films are extracted for RNA which is quantitated by non-competitive, semi-quantitative RT-PCR and normalized to b-actin. Shave biopsies were extracted for total RNA and similarly assayed. The following cytokine mRNAs were assayed and normalized to b-actin: IL-1a, IL-1b, IL-8 and TNFa. In the trial with dilute SLS (0.05%), 7 subjects displayed varying reactions to occlusive patching and on average took up to 4 days to display any clinical reaction. 6 subjects showed increases of 4-to-200-fold in one or more of IL-1a, IL-1b or IL-8 at the SLS site compared to the water site. These increases were rarely sustained for more that 24 hours and were evident before clinical symptoms in at least 4 subjects. In a second trial with 1% SLS, 10 subjects were occlusively patched with SLS and water at two replicate sites for 6 hours and 24 hours. One replicate site was harvested at 6 hours and the cytokine profile compared to clinical symptoms at 24 hours. Results showed that subjects with the most extreme clinical symptoms at 24 hours also had the largest increases in cytokine mRNAs at 6 hours, thus the 6-hour time point was predictive of the 24-hour clinical result. We believe that the application of EGIR tapes and cytokine profiling may allow the prediction of irritant reactions in less than 24 hours.

0108

Immunomodulatory functions of substance P in autoimmune hair loss

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Neuropeptides such as substance P (SP) play a critical role in the cross talk of the skin nervous and immune system, and SP is known to control immune cell functions by acting through the NK-1 receptor. Here, we have studied the role of SP in alopecia areata (AA) an autoimmune disease of the hair follicle characterized by perifollicular inflammatory cell infiltrates. In C3H/HeJ mice induced to develop AA, SP expression assessed by ELISA was markedly reduced in lesional skin as compared to normal skin ($p<0.01$). In addition, RT-PCR analyses showed levels of neutral endopeptidase (NEP), the most important SP-degrading enzyme, to be greatly upregulated in lesional skin, which may explain the low levels of SP found in AA affected skin. Immunofluorescence (IF) staining for NEP and NK-1 revealed pronounced NEP expression by follicular keratinocytes of the outer root sheath, whereas NK-1 was found to be predominantly expressed by intra- and perifollicular T cells and macrophages (MΦ). To test the functional relevance of our findings, we assessed T cell and MΦ numbers in AA lesions of C3H/HeJ mice after treatment with SP or vehicle. Notably, SP significantly reduced the numbers of perifollicular CD4+ and CD8+ T cells ($p<0.05$ and $p<0.01$, respectively) as well as MΦ ($p<0.01$) indicating that SP can modulate the extent of inflammatory cell infiltrates in AA. As we have recently shown that the neurotrophic factor BDNF can induce apoptosis in T cells infiltrating AA lesions by acting via the $p75^{NTR}$ receptor, we assessed BDNF expression in AA skin by IF staining. Interestingly, BDNF was most strongly expressed by NK-1 positive infiltrating immune cells. Thus, SP may control inflammatory cell populations in AA lesions directly or by inducing the release of pro-apoptotic BDNF. Our findings suggest that low levels of SP, possibly due to increased degradation by NEP, contribute to the induction of AA lesions and that exogenous SP can reduce hair follicle damaging cell populations in AA affected skin.

0109

Application of microarray-based data for class prediction in psoriasis

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Psoriasis is a chronic inflammatory skin disease affecting 2% of the population and is believed to be multigenic in origin. There is strong evidence to support an autoimmune process, however, the precise mechanisms of disease induction and progression are not fully understood. We used DNA chips for the identification of significant alterations in gene expression profiles of nine psoriatic patients. Image data was first obtained and analyzed using MAS v5.0, and metrics files were subsequently exported and analyzed using GeneSpringTM v4.2.1 using a non-parametric test with a P-value cut-off at 0.05. Three hundred genes were found to be differentially expressed in psoriatic skin when compared to non-lesional skin. Of these, 202 genes were up-regulated and 98 were down-regulated in psoriatic skin. Functional classification of the differentially expressed sequences reveals that the majority encode structural proteins (KRT15, CDH19, IVL, TGM1), or are involved in cell cycle regulation (RB1, CKS2, CDC37), signal transduction (LYN, SYK, ARGBP2, STAT1) and host immune response (DEFB4, IL1RN, IL4R, SCYA22). Hierarchical clustering showed a distinct gene expression pattern distinguishing lesional from non-lesional skin. Further, we identified a set of 10 genes that can accurately predict psoriasis using the K-nearest-neighbor prediction method. Gene expression profiles of these 10 predictor genes were confirmed using real-time RT-PCR. Subsequent analysis of the predictor gene expression levels in histologically unclassified samples was then used for class prediction of psoriatic vs non-psoriatic skin, and prediction was confirmed by histology. We demonstrate here that predictor gene expression profiles can be employed as a supplemental diagnostic tool for the identification of psoriasis and distinction from other inflammatory skin conditions.

0110

Phenolic-based compounds completely inhibit interleukin-1-induced prostaglandin E_2 production through a mechanism independent of COX-2 or phospholipase A_2 in human fibroblasts

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Prostaglandin E_2 (PGE2) is a potent vasodilator and is known to play a critical role in inflammatory skin disorders. PGE2 production from the substrate arachidonic acid, which is released from membrane glycerophospholipids by phospholipase A2, is controlled by two rate-limiting steps. The first step is catalyzed by cyclooxygenase (COX), which converts arachidonic acid to the intermediate prostanoid PGH2. Two isoforms of the COX enzyme have been identified: cyclooxygenase 1 (COX-1), which is a constitutive enzyme, and cyclooxygenase 2 (COX-2), which is induced by inflammatory stimuli, such as lipopolysacharide (LPS) or the cytokine interleukin-1 (IL-1). The second step of PGE2 synthesis is the terminal conversion of PGH2 to PGE2, which is catalyzed by PGE_2 synthase (PGES). PGES is also induced by the inflammatory stimuli LPS and IL-1. Inhibition of enzymes in the pathway of PGE2 production, such as phospholipase A2 and COX-2, have proven to be beneficial in the reduction of inflammation. However, compounds able to inhibit PGES have yet to be identified. Upon screening a library of phenolic-based compounds, CX-452 and CX-1090 were found to completely inhibit IL-1 induced PGE2 production in human dermal fibroblasts. These effects were not due to the direct inhibition of the phospholipase A2 or COX-2 enzymes. Further, CX-452 and CX-1090 did not affect COX-2 mRNA or protein abundance. They also did not significantly inhibit PGES protein abundance. The action of these compounds on the activity of the PGES enzyme suggests a novel and potentially clinically useful mechanism for inhibiting PGE-2 production.

0111

Pathogenic antigen-specific immune responses to human bullous pemphigoid antigen 2 (hBPAG2) transgenic (Tg) skin in wild type (wt) mice are dependent on CD4+ T cells and are mediated by hBPAG2-specific IgG

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It is hypothesized that IgG autoantibodies in patients with bullous pemphigoid (BP) are pathogenic and that T cells provide crucial signals for their production. Recently, we produced phenotypically normal, C57BL/6 Tg mice that stably express immunoreactive hBPAG2 in murine epidermal BM. To develop and characterize experimental immune responses to hBPAG2, Tg skin was grafted onto gender-matched, syngeneic wt, MHC I -/-, MHC II -/-, or CD4 -/- mice. Median graft survival time (MST) was 28 and 30 days in wt and MHC I -/- mice, respectively; graft loss followed development of antigen-specific IgG in both strains and thus was independent of CD8+ T cell activity. Tg skin placed on previously grafted wt mice (i.e., mice with anti-hBPAG2 IgG) displayed a MST of only 12 days. Direct IF microscopy of Tg skin grafts placed on "immune" wt mice identified in situ deposits of IgG, IgM, and C3 in epidermal BM. Light microscopy studies of such grafts identified vacuolar alterations and granulocyte-rich infiltrates forming just beneath basal keratinocytes like those seen in early lesional skin from patients with BP. Interestingly, Tg skin placed on MHC class II -/- mice demonstrated ongoing survival (> 180 days); these mice consistently failed to develop hBPAG2-specific IgG. CD4 -/- mice had significantly delayed loss of Tg skin grafts (MST=60 days) that again followed the development of hBPAG2-specific IgG. Adoptive transfer of hBPAG2-specific CD4+ T cells to CD4 -/- mice bearing Tg skin prompted graft loss only subsequent to development of anti-hBPAG2 IgG (rather than immediately following the administration of antigen-specific CD4+ T cells). In sum, these findings indicate that loss of Tg skin in these grafting paradigms is mediated by antigen-specific IgG. hBPAG2 in Tg skin elicits specific and pathogenic immune responses in naive wt mice that mimic the consequences of immune responses to this protein in patients with BP.

0112

Alopecia areata phylogenetic tissue arrays

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Alopecia areata is a relatively common human autoimmune skin disease that targets anagen stage hair follicles. Clinically and pathologically similar diseases occur in mice, rats, dogs, and other species. Variability between individuals in outbred species suggests that either there are many factors involved in the disease or that several diseases with similar features are lumped together. Creation of tissue arrays consisting of 49 punch biopsies on a single microscope slide provides a tool to directly compare the histologic features between cases. Parallel slides from cases in other species permits phylogenetic comparisons. Immunohistochemical analyses of these tissues provides ways to analyse immediately many samples tested with the same antibody at the same time. We found that although patterns are similar between individuals as well as between species, there is more variability than generally reported. These tissue arrays will be available to investigators later this year through the National Alopecia Areata Repository (hppt://www.AlopeciaAreataRegistry.org).

0113

Global gene expression analysis identifies a novel apoptotic pathway in cutaneous lupus erythematosus

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The molecular basis for the initiation and mechanisms of progression in lupus are not well understood. However, it is becoming increasingly clear that the pathogenesis of many human diseases is associated with a dysregulation of apoptotic pathways. Apoptosis serves to remove superfluous, damaged or harmful cells in a wide variety of physiological contexts and is crucial for tissue morphogenesis and for maintaining cellular homeostasis. Autoantibodies in sera from LE patients have been shown to recognize antigens present within apoptotic blebs, suggesting that apoptotic cells serve as a source of autoantigens and play a major role in the development of this disease. We used DNA chips to search for significant alterations in the gene expression profiles of 6 LE patients. Ninety-five genes were found to be differentially expressed in LE skin relative to non-lesional skin. Hierarchical clustering analysis demonstrated a distinct gene expression pattern distinguishing affected from non-affected skin. Of the 95 differentially expressed genes (DEGs), 84 genes were up-regulated and 11 were down-regulated in LE skin. Classification of the 95 DEGs based on ontology revealed that the majority are involved in apoptosis, host response mechanisms and signal transduction. Interestingly, we found an up-regulation in expression of genes encoding both pro-apoptotic mediators (FAS, CASP10 and GZMB) and anti-apoptotic mediators (TRAF1 and TNFAIP3) in cutaneous LE lesions. Our preliminary data also suggests the execution of a novel apoptotic pathway involving the expression of 2'-5'-oligoadenylate synthetase (OAS) genes and proteins in LE skin lesions, which might contribute to the induction and/or maintenance of the autoimmune state.

0114

***Staphylococcus aureus* induces TNF-α expression in primary cultured human keratinocytes**

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Bacterial infections of the skin are clinically recognized through inflammation, suggesting a pro-inflammatory immune response. Yet little is known regarding the role of skin cells in the pro-inflammatory response to invading bacteria. Toward this regard, we studied the role of human keratinocytes in the host immune response to infection with *Staphylococcus aureus*, the most common cause of cutaneous bacterial infections. Particularly, we focused on investigating the involvement of pro-inflammatory cytokines in *S. aureus* infection. Specifically, we have characterized the ability of *S. aureus* to induce the cytokine TNF-α,an essential mediator of inflammatory and immunological reactions during host defense, using human keratinocytes. TNF-α mRNA synthesis of infected keratinocytes was examined using Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR). Concurrently, TNF-α protein expression was studied using Enzyme Linked-Immuno-Sorbent Assay (ELISA). Previously we have shown that *S. aureus* adheres rapidly and efficiently to human keratinocytes, followed by a slow but persistent invasion (~2-4% in 4 hr) and subsequent intracellular replication. TNF-α mRNA expression was induced at 6 hr and 24 hr after *S. aureus* infection, and decreased at 48 hr post infection. Similarly, TNF-α protein levels were increased 2-fold and 8-fold at 6 hr and 24 hr after *S. aureus* infection, respectively. Infection of keratinocytes with several other strains of *S. aureus* showed similar TNF-α induction. Entry by bacteria into human keratinocytes is critical for increased TNF-α secretion. In conclusion, our study showed that human keratinocytes secrete TNF-α in response to *S. aureus* invasion. Expression of TNF-α by keratinocytes in response to *S. aureus* infection is recognized as one of the early events following the infection by variety of human pathogens. TNF-α is an important pro-inflammatory cytokine not only because it plays a crucial role in pathogenic events accompanying bacterial invasion, but it also can function in synergy with other cytokines that are involved in inflammatory reactions.

0115

Increased expression of CD132 and multiple IL-2 family receptors in psoriasis vulgaris

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The IL-2 family receptors (IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R) have not been well characterized in psoriasis. This family is defined by its shared use of the common gamma chain (CD132) for signal transduction. We used RT-PCR, FACS and immunohistochemistry (IHC) to examine expression of these receptors in skin biopsies. CD132 mRNA was upregulated in lesional versus nonlesional skin ($p<0.02$). IHC for CD132 showed expression confined to infiltrating leukocytes with no detectable expression on keratinocytes. FACS identified CD132 on the surface of T cells and dendritic cells in psoriatic skin with highest expression on activated dendritic cells. T cells in lesions expressed higher levels of CD132 than those in peripheral blood. IL-2Rα, IL-4R, IL-7R and IL-21R mRNA were also upregulated in psoriasis lesions (p values<0.02). IL-15R mRNA was consistently detected in lesional and nonlesional skin. FACS demonstrated upregulation of IL-2Rα, IL-7R and IL-15R on the surface of cutaneous T cells when compared to those in peripheral blood. FACS also showed that dendritic cells expressed IL-15R, upregulated IL-2Rα and IL-7R in their activated subpopulation and were the only cell type studied to express IL-4R. IHC suggested co-expression of IL-2Rα and IL-7R with CD132. IHC for IL-15 stained keratinocytes as well as the inflammatory infiltrate. Although IL-9R mRNA showed a downward trend in lesional skin, IHC indicates possible expression on psoriatic blood vessels. IHC for IL-21R produced intense keratinocyte staining but this is unlikely to represent a functional receptor in the absence of co-expression with CD132. Interestingly, we were unable to detect IL-21 mRNA in lesional or nonlesional skin. These data suggest a complex biology of the IL-2 family receptors in psoriasis and have important implications for receptor-directed therapies.

0116

Corticosteroids block acantholyis by strengthing cell-cell adhesion

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Corticosteroid therapy transformed pemphigus vulgaris (PV) from a fatal disease to a highly treatable one. Although corticosteroids have well characterized immunosupressive properties, the precise mechanism of these agents in pemphigus has remained elusive. We used an in vitro model of PV antibody mediated acantholysis to examine the mechanism through which corticosteroids inhibit acantholysis. Pretreatment with methylprednisolone blocked PV antibody mediated acantholysis of human epidermal keratinocytes. We measured the effect of corticosteroid treatment on the function, levels, and localization of desmosomal components in keratinocytes. Using a mechanical cell dispersion assay, we found that corticosteroids caused a dramatic strengthening of keratinocyte cell-cell adhesion. This change in adhesion was associated with increased levels of desmoglein 3 protein and the assembly of desmoplakin containing foci at sites of cell-cell contact. These results suggest that strengthening of keratinocyte adhesion may contribute to the therapeutic action of corticosteroids in PV and other vesiculobullous diseases.

0117

Mast cells induce aberrant angiogenesis in a model of rheumatoid arthritis

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Auto-antibodies against glucose-6-phosphate isomerase induce T cell-independent arthritis in mice. As mast cells are important amplifiers of T cell-dependent and T cell-independent inflammation, and can produce mediators involved in angiogenesis, we studied whether mast cells influence neoangiogenesis, a hallmark of psoriasis arthritis and other chronic inflammatory diseases. Comparing wild type mice, mast cell-deficient kitW/W-v mice and mast cell-reconstituted kitW/W-v mice, we found that mast cells were essential for pannus formation and integrin αvβ3-expression and neoangiogenesis during chronic inflammation. This was confirmed by in vivo-quantification of activated αvβ3 integrin using radio-labeled RGD-peptide and positron emission tomography. In consequence, therapeutic mast cell-degranulation inhibitors also prevented αvβ3-activation, angiogenesis and tissue destruction. As these data unravel a critical role of mast cells and mast cell-derived mediators in chronic inflammation, inhibition of mast cell-degranulation may not only attenuate acute arthritis but also the late damages of psoriais arthritis resulting from neovasularization and scar formation.

0118

Psoriasis-like inflammatory skin disease in mice with epidermis-specific-ablation of IκB kinase 2

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Cutaneous inflammation involves interactions between several cell types that populate different compartments of the skin. The role of epidermal keratinocytes in its pathogenesis is not well characterised. Using conditional gene targeting we have ablated the gene for IκB kinase 2 (IKK2) specifically from the epidermis of mice. Deletion of IKK2 attenuates cytokine stimulated NFκB activation but does not stimulate proliferation or inhibit differentiation of keratinocytes. After exposure to the extramaternal environment, newborn mutant mice develop a severe inflammatory skin disease that closely resembles human psoriasis. We show that the pathogenesis of this disease does not require alpha/beta T lymphocytes but depends on the presence of the type I receptor for tumour necrosis factor (TNF). Our results suggest that epidermal keratinocytes can control skin inflammation and are capable of initiating a psoriasis like skin disease in mice. The pathogenesis of this disease involves interactions between epidermal keratinocytes and dermal cells and depends on TNF- mediated cell-cell communication.

0119

HLA pocket polymorphisms define functionally-active desmoglein 3 sequences in pemphigus vulgaris

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Susceptibility to pemphigus vulgaris (PV) is correlated with the HLA alleles DRB1*0402 and DQB1*0503, but the mechanism of HLA mediated susceptibility is not understood. While disease is ultimately mediated by anti-desmoglein 3 (Dsg3) antibodies, an initial T cell response restricted to HLA DRB1*0402 or DQB1*0503 is presumably required to generate anti-Dsg3 autoantibodies. There is no consensus regarding T cell epitopes relevant for disease induction and/or propagation. We have defined a DRB1*0402 motif to deduce Dsg3 sequences able to form MHC II-peptide complexes. Top scoring peptides were synthesized and screened for functional activity by proliferation assays with patient PBMC. T cell reactivity to 4 peptides was observed. Response to at least one peptide was detected in 5/9 patients that typed as DRB1*0402. Two epitopes, Dsg3 809-823 and 962-973 are contained within the cytoplasmic region of Dsg3, while Dsg3 190-204 and 341-355 map to the extracellular domains. All stimulatory sequences (Dsg3 190-204, 341-355, 809-823, and 962-976) were shown to directly complex with purified DRB1*0402/DRA1*0101 molecules in a competitive binding assay. Two peptides able to bind DRB1*0402 did not stimulate T cell response in any patients (or HLA matched controls), suggesting T cell tolerance to at least some portions of the Dsg3 self protein. We also show a response within one patient to two peptides (Dsg3 341-355 and 190-204) and another to three peptides (Dsg3 962-973, 190-204, and 809-823). Our data suggest that the intracellular and extracellular portions of Dsg3 harbor disease relevant T cell epitopes and that multiple epitopes may be operative in the autoimmune response in an individual patient. These findings may be of clinical relevance regarding disease initiation and subsequent epitope spreading.

0120

Construction of human TNF-α expression vectors under the control of TNF-α promoter polymorphisms

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Tumor necrosis factor-α (hTNF-α) is involved in apoptosis and immunomodulation. Studies have shown that the hTNF-α promoter polymorphism, -308A, results in increased transcription following UVB irradiation in the presence of interleukin-1. This promoter polymorphism is prevalent in subacute cutaneous lupus erythematosus (SCLE) and may represent a mechanism contributing to photosensitive dermatitis and the development of autoimmunity. To investigate the role of hTNF-α expression from the -308A TNF-α promoter polymorphism, we constructed vectors expressing hTNF-α under the control of the wild type promoter (-308G) and -308A polymorphic promoter. A multistep cloning strategy was utilized to create these constructs, and the vector sequences were confirmed by sequencing. We will use these constructs to investigate whether the -308A promoter polymorphism of human TNF-α affects apoptosis as well as translocation of intracellular antigens to the cell surface. Furthermore, we plan to investigate whether TNF-α-induced apoptosis leading to the presentation of intracellular antigens results in the development of autoimmunity seen in cutaneous LE. These studies will contribute to our understanding of the pathogenesis of SCLE and may direct research toward potential treatments for SCLE.

0121

Gene expression profiling in vitiligo by cDNA microarray analysis provides support of immune-mediated mechanisms of disease

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Vitiligo is a debilitating skin disease with areas of pigment loss, affecting one or two of every 100 people. The cellular and molecular mechanisms leading to the destruction of melanocytes in this disorder have not yet been elucidated. The three prevailing theories of the pathogenesis of vitiligo are the immune hypothesis, the neural hypothesis, and the self-destruct hypothesis. According to the available data, it is likely that the loss of epidermal and follicular melanocytes in vitiligo may be the result of several different pathogenetic mechanisms. Microarray technology allows for simultaneous screening of a wide range of genes that may be involved in the pathogenesis of multigenic diseases such as vitiligo. In our studies, DNA chips were used for the identification of significant alterations in the gene expression profiles of 7 vitiligo patients. Image data was first obtained and analyzed using MAS v 5.0, and metrics files were subsequently exported and analyzed using GeneSpringTM v4.2.1 using a parametric test with a P-value cut-off at 0.05. Hierarchical clustering showed a distinct gene expression pattern distinguishing affected from unaffected skin. We report 75 differentially expressed genes (DEGs) when comparing affected with unaffected skin. Of these, more than 40 were up-regulated in lesional skin and more than 30 were down-regulated in lesional skin. Functional classification of the DEGs show that the majority are involved in signal transduction (GLI, PLCG2, CAMP-GEFII), immune response (CD86, INDO, LIFR, TNFAIP3), and DNA replication and repair (RPA3, RECQL). In addition, a small set of DEGs encodes oncogenes. This microarray-based expression data defining vitiligo was confirmed by real time RT-PCR and further functional analysis with a larger data set of skin samples are currently undertaken.

0122

Differential regulation of inflammatory signaling pathways by retinoids and corticosteroids in human sebocytes

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The combination of 13-*cis* retinoic acid (13cRA) and corticosteroids has been shown to be markedly effective in severe inflammatory acne, such as acne fulminans. On the other hand, we and others have currently shown that human epithelial cells are likely to modulate inflammatory signals by intrinsic mechanisms. The toll-like receptor (TLR) / CD14 pathway, which is involved in the innate immunity by being able to recognize microbial components, and cytokine /chemokine synthesis has been implicated as such mechanisms. We address here the influence of 13cRA, hydrocortisone and the microbial components lipopolysaccharide (LPS), and lipoteichoic acid (LTA) on TLR2 and TLR4 mRNA and protein expression (TaqMan quantitative PCR and immunocytochemistry, respectively) as well as interleukin-1α (IL1α), IL6, and IL8 mRNA expression and synthesis (TaqMan PCR and ELISA). Phorbol 12-myristate 13-acetate (PMA) served as positive control. SZ95 sebocytes expressed TLR2 and TLR4, IL1α, IL6, and IL8 on mRNA and protein levels. LPS enhanced TLR2 and cytokine / chemokine expression, but not TLR4 (n=7, $p<0.05$), while LTA barely exhibited any effect. 13cRA suppressed IL1α and IL6 synthesis but has no effect on TLR or IL8 expression. Hydrocortisone suppressed TLR4 and cytokine expression but did not affect TLR2 expression (n=7, $p<0.05$). PMA increased TLR and cytokine / chemokine expression dramatically (n=7, $p<0.001$). The present work provides first evidence that retinoids and corticosteroids exhibit distinct anti-inflammatory activities on epithelial skin cells and, therefore, the retinoid / corticosteroid combination may be meaningful in severe inflammatory skin diseases.

0123

Immunization with pemphigus anti-idiotype F(ab)'$_2$ fragments elicit anti-epithelial antibodies in adult Balb\c mice

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The immunization with anti-idiotype antibodies would elicit antibodies capable to recognize certain epitopes like the original antibody does, such recognition would be elicited by internal images. The major goal of present study was elicit anti-epithelial antibodies in adult Balb\c mice by Cre anti-idiotype antibody from pemphigus foliaceous previously described. For this purpose six adult Balb\c mice were immunized weekly with Cre anti-idiotype F(ab)'$_2$ fragments. IgG anti-idiotype was digested by pepsin, and F(ab)'$_2$ fragments were purified by gel filtration chromatography in Sephacryl S-300. All immunized animals developed anti-epithelial antibodies, starting at the 4th week. Fine specificity by ELISA demonstrated anti-desmoglein 1 antibodies in three animals after two months, this animals developed blisters, and their skin biopsies exhibited intraepidermal blisters and acantolysis. Direct immunofluorescence showed IgG deposition along inter-cellular spaces of epidermal cells. Controls were negative. In conclusion, anti-epithelial antibodies can be elicited by internal images of pemphigus anti-idiotypes.

0124

Insulin-like growth factor-1 stimulates bcl-2-like gene expression and protects against dexamethasone-induced apoptosis in neonatal mouse skin

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Growth factors and hormones are believed to play important roles in skin growth, differentiation and tumor formation. Insulin-like growth factor 1 (IGF-1) has been identified as a survival factor in many tissues including the skin, but the molecular mechanism of IGF-1 in skin carcinogenesis is not completely understood. Using RNA differential display, a 358-bp message has been identified in the skin of IGF-1-injected mice but not in controls. Confirmation of expression of this gene in the skin by ribonuclease protection assay showed that its expression is dependent on the treatment of IGF-1. Using rapid amplification of 5' cDNA ends, we have successfully cloned a 3473-bp full-length gene (GenBank accession No. AY170344), which has 99% sequence homology to a bcl-2-like gene. The latter has been identified as a proto-oncogene in several murine myeloid cell lines. In addition, IGF-1 can prevent epidermal keratinocytes against dexamethasone- (DEX-) induced apoptosis, based on the findings that DNA laddering was present in the control mice but not in IGF-1- and DEX-treated ones. Further, using a photometric enzyme-linked immunoassay to quantitate keratinocyte death, we found that after addition of DEX, the amounts of cytoplasmic histone-associated DNA fragments were not significantly ($p > 0.05$) different in IGF-1-treated cells compared with untreated cells but significantly ($p < 0.001$) higher amounts of DNA fragmentation were observed in vehicle-treated compared with untreated cells. Our current data demonstrated that IGF-1 plays an anti-apoptotic role in the proliferation of mouse skin and this, at least in part, may be mediated through expression of bcl-2.

0125

High frequency of BRAF mutations in melanocytic skin lesions of various dignity

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Genes of the RAF family are components of intracellular signal transduction pathways. BRAF encodes a kinase that mediates cell growth and malignant kinase pathway activation, which can be target of a specific therapy. A recent study reported about the T1796A point-mutation in exon 15 of the BRAF gene in a high frequency in melanoma cell lines and in 6 of 9 primary malignant melanomas (MM). In order to evaluate the role of the T1796A BRAF mutation in tumorigenesis of malignant melanomas we screened 303 melanocytic skin lesions for the presence of the specific BRAF mutation including malignant melanomas, malignant melanomas in situ, various types of melanocytic nevi, Spitz nevi and malignant melanomas with an underlying nevus by single strand conformational polymorphism (SSCP)and direct DNA sequencing. The frequency of the mutation in malignant melanomas was considerably low (28/97), but the specific nucleotide change could be detected in several non-malignant melanocytic skin lesions (39/187). Papillomatous nevi showed the highest frequency of the T1796 A mutation (20/27), whereas in Spitz nevi (0/63) and blue nevi (0/19) the mutation could not be detected at all. Combined lesions of malignant melanomas and nevi showed the mutation both in the microdissected nevus (3/14) and melanoma cells or both lesions had the wild-type (10/14) allele, whereas in only one case the mutated nucleotide could be identified solely in the malignant melanoma part. We screened 303 (including 94 laser-capture microdissected tissue samples) for the presence of T1796A. As the specific mutation of BRAF was found in similar frequency in both malignant and benign lesions, other factors are supposed to play a role in oncogenesis of malignant melanomas.

0126

Advances in phytotherapy of skin cancer

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Medicinal herbs are significant source of synthetic and herbal drugs. Before the availability of synthetic drugs, man was completely dependent on herbs as healthcare system. Medicinal herbs have been on the forefront whenever we talk about anticancer remedies. Vinblastine, Vincristine, Podophyllotoxin and Taxol are reputed drugs in the field of chemotherapy. Psoralens in association with UV light has been employed for the treatment of skin cancer. Medicinal plants have not been exploited to their potential, as far as treatment of skin cancer is concerned. The paper reviews latest developments in phytotherapy of cancer. Silymarin (Silybum marianum) has shown protective effects against photocarcinogensis in mouse skin model. The drug offered significant protection against UVB-induced carcinogensis. Curcumin, the active constituents of Curcuma longa, effectively inhibited metastasis of melanoma cells in animal studies. Laboratory and animal studies have shown that active constituent of Rosmarinus officinalis, carnosolic acid and ursolic acid inhibited the skin tumorigensis. Pheophorbide-A inhibited skin tumor promotion in ICR mouse. Cassiamin isolated from Cassia siames demonstrated chemoprotective effects against mouse skin carcinogensis. Salvia officinalis and Withaferin-A obtained from Withania somnifera inhibited skin tumors in DBMA (dimethyl benzanthracene) induced complete carcinogensis. We conclude that medicinal plants and natural products have demonstrated significant results in animal models. The phytochemicals with potential anticancer activity should be screened for large-scale clinical trials.

0127

Utility of a microarray scanner to enhance intra-operative margin control during Mohs micrographic surgery

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Mohs micrographic surgery for the removal of squamous and basal cell carcinoma relies on the intra-operative examination of hematoxylin and eosin (H&E)-stained frozen biopsy sections during the surgical procedure to confirm clearance of the tumor margin. In certain cases, ambiguity can arise in evaluating the H&E sections due to the presence of inflammatory infiltrate or ambiguity in judging the morphology of tiny nests of cells that may or may not be malignant. In this study we have employed the use of a rapid immuno-labeling procedure in conjunction with a microarray scanner as an adjunct to the H&E sections in evaluating the clearance of the tumor margin in ambiguous cases. The scanning method provided a complete image of the entire Mohs surgery section, including histological details. This eliminated the need to draw a freehand Mohs map. We were able to generate scanned images of the entire Mohs section for viewing within 20 minutes of receiving the frozen section. Our wide-spectrum keratin antibody clearly labeled the epidermis, sebaceous glands, eccrine and apocrine glands, hair-follicles and the tumor. Double-labeling using a wide spectrum keratin and keratin 19 allowed us to clearly distinguish small nests of tumor cells from non-malignant cells that appeared ambiguous on the H&E slide. The rapid immuno-labeling and scanning methodology can serve as valuable adjunct to the evaluation of the H&E sections currently used in Mohs surgery. The scanning methodology is extremely helpful in cases of ambiguity, to identify hidden tumor cells. Thus, this novel application of a microarray scanner has a significant and practical use in clinical settings such as that in Mohs micrographic surgery.

0128

Inducible, haploinsufficiency of PTEN in transgenic mouse skin is a lesion-eliciting event during activated Ha-ras-mediated papillomatogenesis

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To investigate the role of PTEN tumour suppressor gene failure in Ha-ras-mediated transgenic mouse skin carcinogenesis, mice containing a floxed PTEN allele were mated to HK1.ras mice carrying an inducible cre recombinase expressed from the K14 keratin promoter [K14.CreP]. On topical application of the progesterone agonist Ru486, cre recombinase acts to ablate exon 5 of the floxed PTEN allele(s) and inactivate function exclusively in keratinocytes, including stem cells. HK1.ras line 1276 expresses v-Ha-ras in transit amplifying keratinocytes and suprabasal cells to elicit an initiated epidermis, which does not exhibit spontaneous papillomas, despite an ear tag wound promotion stimulus. On treatment with Ru486, HK1.ras 1276 mice heterozygous for the K14/PTEN floxed allele developed novel benign tumours at wounded [ear tag] sites by 3-4 weeks, whilst 1276 siblings homozygous for the K14/PTEN allele rapidly developed highly keratotic ear lesions, within 2-3 weeks of treatment, apparently independent of wound promotion, and displayed a dorsal skin hyperkeratosis. These data demonstrate that inducible haploinsufficiency and reduction in the function(s) of PTEN is sufficient to cooperate with wound associated Ha-ras-mediated papillomatogenesis, displaying an apparent lesion-eliciting event. The highly keratotic nature to the lesions following complete PTEN ablation highlights a disruption to the ordered differentiation kinetics of initiated/benign lesion keratinocytes and suggests a response mediated by failures in cell-cell adhesion and/or adhesion signalling.

0129

A mitochondrial genome-wide screen for mutations in UV-induced tumors in mouse skin

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The mitochondrial genome of most North American laboratory mouse strains is a 16,295 bp circular DNA molecule that encodes 13 polypeptides involved in oxidative phosphorylation and a complete set of tRNAs and rRNAs required for their translation on mitochondrial ribosomes. The accumulation of homoplasmic mitochondrial DNA (mtDNA) point mutations has been observed in various human malignancies including our labs findings of these mutations in non-melanoma skin cancers. We sought to identify the potential role of mtDNA point mutations in the pathogenesis of cutaneous tumors in an animal model. We excised cutaneous tumors induced in the hairless mouse by repetitive doses of UVA and UVB light delivered over ten weeks. We designed and optimized oligonucleotide primer pairs that span the entire mouse mitochondrial genome to produce 51 amplicons with a minimum of 15 percent overlap. The PCR products amplified from tumor DNA were mixed with PCR products amplified from wild type mouse mtDNA. Thermal denaturation of these mixed mtDNA amplicons was followed by slow cooling, allowing for the formation of heteroduplexes if the amplified DNA fragments contain genetic variation. The WAVE system (Transgenomics) is a sensitive, high throughput, column-based method used to detect DNA variation as small as a single base change within the amplicon by denaturing high-pressure liquid chromatography (DHLPC). The mixed PCR products were applied to the HPLC column at a temperature that partially denatures the strands and the DNA was subsequently eluted using increasing concentrations of acetonitrile and measured with UV light detection. Using this method, homoduplex DNA produced a single peak and was resolved from heteroduplex DNA which produced multiple peaks. We are currently sequencing the amplicons that produced heteroduplex peaks for identification of the specific nucleotide changes. This method allows for convenient screening of the entire mitochondrial genome for mutations potentially arising in cutaneous neoplasia.

0130

Withdrawn.

0131

Tumor suppressor and motility functions of p16INK4A growth arrest in normal and premalignant epidermis and oral epithelium

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A high frequency of mutation, deletion, and promoter silencing of the p16INK4A (p16) gene in premalignant dysplasias and squamous cell carcinomas (SCC) of epidermis and oral epithelium has identified p16 as a tumor suppressor. The point during neoplastic progression at which p16 is expressed and impedes formation of an SCC has remained unknown. Upregulation of p16 expression has been found to cause senescence arrest and to limit the lifespan of normal human keratinocytes in culture, suggesting the possibility that excessive or spatially abnormal cell growth in vivo may trigger p16 expression. We examined 77 archival epidermal and oral mucosal biopsy specimens to test three increasingly focused hypotheses about the setting in vivo in which p16 expression occurs. We found that p16 expression does not result from chronic hyperproliferation but does occur heterogeneously in cells of dysplastic and carcinoma in situ (CIS) lesions, most consistently by cells at the invasive fronts of early SCCs. Such cells coexpressed p16 and laminin 5 gamma 2 (lam5g2), the latter identified previously by others as a marker of early invasion in carcinomas. Cells in normal keratinocyte cultures also coordinately upregulated p16 and lam5g2, associated with increased motility. Keratinocytes at the edges of experimental wounds made in confluent cultures also upregulated p16 and lam5g2, accompanying migration to fill the wound. Some cell lines that were p16-deficient or engineered to resist p16 arrest secreted lam5g2 and were motile. Thus, p16 expression does not cause but, rather, is induced coordinately with lam5g2. These results identify the point at which p16 acts as a tumor suppressor in vivo and suggest that normal keratinocytes may use the same mechanism to generate non-proliferative, motile cells for wound repair.

0132

Optimization of DNA microarray hybridization methods to detect cancer-related changes in gene expression in cultured human keratinocytes

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The validity and reliability of DNA microarray hybridization for characterizing gene expression has remained uncertain. We have compared gene expression profiles of four normal primary keratinocyte and five squamous cell carcinoma (SCC) cell lines of human oral epithelial and epidermal origin. Fluorescent cRNA samples were prepared from RNA isolated from cells growing exponentially in GIBCO ker-sfm medium and hybridized to Affymetrix U95Av2 arrays. Four cell lines were also analyzed on the Affymetrix U133 Set and on the Amersham/Motorola CodeLink Bioarray. Affymetrix hybridization signals were normalized to a target intensity of 100 and analyzed using MASv5.0, dChip and RMA programs. Duplicate samples and repeat scans of the same hybridization often varied by 2-fold or more for signal calls less than 30, so genes for which no sample yielded a signal higher than 30 were excluded from analysis. 58 genes were identified for which expression levels of normal vs. SCC cells (U95Av2/MASv5.0) differed more than 3-fold with P values (t-test) of 0.015 or better. These 58 included genes encoding 10 transcription factors, 12 membrane receptor signal pathway proteins, 7 adhesion proteins, and 5 RNA binding proteins. Real-time PCR confirmed the results for 25 of 28 genes tested (89%). Results for the U133 Set and U95Av2 were similar and the CodeLink chip confirmed 70% of PCR-verifiable genes identified by U95Av2. MASv5.0 was more sensitive than dChip or RMA in detecting PCR- verifiable differences. Our study has determined systematic methods for identifying verifiable differences in gene expression using multiple platforms, thereby providing the basis to search for genes involved in keratinocyte aging, senescence, immortalization, and malignant transformation.

0133

Specificity of p16-enforced growth arrest and Id1 action in keratinocyte senescence

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The expansion potential in culture of keratinocytes and several other epithelial cell types is limited by a mechanism that culminates in expression of the G1/S cdk inhibitor p16INK4A (p16). We have sought to identify exogenous molecules that might arrest keratinocyte growth by activating the same p16-dependent mechanism. We had reported recently that normal keratinocytes engineered to evade p16- and p53-dependent growth arrest mechanisms, as well as a premalignant keratinocyte line that had undergone deletion and mutational loss of the p16 and p53 loci, bypass the normal replicative lifespan limit and continue to divide for a substantial extended lifespan until halted by a short telomere crisis. P16- and p16-/p53-deficient keratinocyte lines and an Id1-overexpressing, extended lifespan keratinocyte line were compared to normal keratinocytes for their sensitivity to a variety of agents that inhibit keratinocyte growth in the GIBCO ker-sfm medium system. We tested the potential involvement of p16 by immunocytology, flow cytometry, and p16 promoter-reporter activity assays. TPA, TGF-beta, serum, IFNgamma, and TNFalpha proved equally inhibitory to normal, p16- and p16/p53-deficient, and Id1-overexpressing cells. P16 levels and promoter activities were not altered significantly by treatment with any of these agents. Senescing normal keratinocytes exhibited p16 expression and arrested in G1, whereas keratinocytes engineered to express a p16-resistant cdk4 senesced with many cells in G2. The latter resembled the behavior of skin fibroblasts, which arrest at senescence from a p53-dependent p21cip1 induction. These results demonstrate that p16 induction in keratinocytes is not a generalized stress response to many types of growth inhibitory signals. They suggest, instead, that p16 induction has a more specialized role as a senescence mechanism in culture and tumor suppressor mechanism in vivo.

0134

p53 regulation is abnormal in xeroderma pigmentosum group E diploid human fibroblasts

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The tumor suppressor p53 is crucial for maintaining cellular homeostasis and genome stability after DNA damage. Mutations in the DDB2 gene give rise to the E subgroup of xeroderma pigmentosum. Although p53 itself and its downstream-regulated genes have been extensively investigated, little is known of an initial enhancement of the p53 protein level that leads to apoptosis and cell cycle arrest. Moreover, the functions of the DDB2 gene product are still unclear. We report here that the DDB2 gene and its protein product regulate p53 protein levels and hence p53-mediated responses to DNA damage.

0135

Expression of chemokine receptor CXCR4 in basal cell carcinoma

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Beside the well-known role to regulate leukocyte trafficking, chemokines and their receptors also play important roles in the regulation of mitosis, apoptosis, survival and angiogenesis, which may enhance tumor growth or metastasis. Herein we investigated the possible involvements of chemokines and chemokine receptors in the carcinogenesis of basal cell carcinoma (BCC), the most common human cancer with unique clinical behaviors featuring local invasion and extremely rare metastasis. Real-time quantitative RT-PCR was used to measure the expression levels of several chemokine receptors (CXCR4, CCR7, CCR10) in various human skin cancer cell lines including BCC, squamous cell carcinoma and melanomas. The expression level of CXCR4 in BCC cell line was ~64-fold more than in other cancer cell lines and normal controls (cultured human keratinocytes and fibroblasts). The expression of CXCR4 in BCC cell line was further confirmed using conventional RT-PCR, immunofluorescent staining and western blotting. Immunohistochemistry studies using tissue samples from BCC lesions showed heterogeneous CXCR4 expression in 18 out of 26 (~69%) specimen. The up-regulation of CXCR4 in BCC suggests its possible role in tumor growth or invasion. Transgenic and xenograft studies are currently under way to determine whether overexpresson of CXCR4 in BCC may alter tumor behavior in nude mice.

0136

Increased expression of p21 and p27 in spindle cell and epithelioid cell nevi (Spitz nevi) in the absence without increased expression of oncoproteins distinquishes these nevi from malignant melanoma

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Spitz nevi usually occur in children and are biologically benign. However, because they may have significant cytologic atypia in some cases the differentiation from malignant melanoma is difficult. Although immunohistochemical markers have not been useful in differentiating these two entities, we wanted to determine if a battery of markers for cyclin dependent kinase inhibitors (CDKI)s and oncoproteins might help. We examined histologic sections from thirty primary malignant melanomas (MM), twenty Spitz nevi using a battery of immunohistochemical stains including cyclin-D-1, c-myc, H-ras, p16, p21, p27, and p53. All Spitz nevi showed +3 nuclear staining for p21, +1-3 nuclear staining for p27, +1 nuclear staining for p16 in two lesions and +1 cytoplasmic staining for H-ras in three lesions. Cyclin D1, c-myc, and p53 were all negative. Although some of the MMs showed a similar pattern of staining for p21 and p27, the melanomas that did, all showed increased expression of one or more the following; cyclin D1, c-myc, and p53 and in some cases also showed higher levels of expression of H-ras and/or p16. A Spitz nevus would not be favored in the absence of significant expression of p21 and co-expression of p27. Although this pattern of expression of CDKIs would not eliminate all cases of MM, addition immunohistochemical stains for cyclin D1 and/or c-myc were positive in these MMs and not in any Spitz nevi.

0137

Stat3 is required for survival of epidermal keratinocytes against apoptotic stimuli and for skin cancer development despite its down-regulation by mutagens

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A growing number of studies have demonstrated that Stat3 is involved in cancer development, although the underlying mechanism is not completely understood. To investigate the role of Stat3 in skin carcinogenesis, we took advantage of keratinocyte-specific Stat3 deficient (K5.Stat3-/-) mice. The epidermis of these mutant mice exhibited larger number of apoptotic keratinocytes upon UVB irradiation or DMBA treatment than that of wild-type mice, and transfection of Stat3 gene normalized the survival of their keratinocytes, indicating that Stat3 is required for protecting keratinocytes from apoptosis. Interestingly, in wild-type keratinocytes, UVB irradiation resulted in down-regulation of Stat3 and its downstream molecule, Bcl-xL. Stat3 overexpression via gene transfer further desensitized wild-type epidermis to UVB-induced apoptosis. These results suggest that down-regulation of Stat3 and Bcl-xL partly contributes to keratinocyte apoptosis of wild-type mice when treated with UVB. Furthermore, requirement of Stat3 and Bcl-xL for skin tumor development was clearly shown by the finding that no skin tumor arose in K5.Stat3-/- or K5.Bcl-xL-/- mice, respectively, through the two-stage protocol of DMBA plus TPA. In sum, we conclude that Stat3 and its downstream Bcl-xL not only function as anti-apoptotic molecules to give rise to skin tumor but also are down-regulated by genotoxic stimuli, including UVB or chemical carcinogens, so that they reduce the future risk of carcinogenesis.

0138

Molecular mechanism of Sphingosine-1-phosphate receptor Edg isoform mediated bimodal regulation of mouse malignant melanoma B16 cell migration and invasion

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Sphingosine-1-phosphate (S1P) is a unique lysophospholipid mediator that potently inhibits migration and invasion of certain tumor cells. It has remained unknown whether the inhibitory effects of S1P are mediated through either one of the Edg family G protein-coupled S1P receptors or by proposed intracellular action of S1P. We investigated molecular mechanisms for S1P inhibition by using Boyden chamber migration and invasion assay techniques and pull-down assays for quantification of GTP-bound active forms of small GTPases in B16 mouse melanoma cells. B16 cell migration across fibronectin-coated porous membrane and invasion through Matrigel layer were inhibited by S1P and less potent Edg5/S1P2 receptor agonists, dihydro-S1P and sphingosylphosphorylcholine, with expected relative potencies. The S1P-selective antagonist JTE013 completely abolished the responses to these agonists, as well as the inhibition by the S1P precursor sphingosine, which is inactive as an agonist for the S1P receptors, indicating that the sphingosine effects were mediated via S1P2 stimulation, most likely by S1P that was converted from sphingosine. S1P induced inhibition and activation, respectively, of small GTPases Rac and RhoA in B16 cells, which were abrogated by JTE013. Adenovirus-mediated expression of a dominant negative Rac mutant, N17Rac, mimicked the inhibitory effects of S1P, whereas inactivation of RhoA by C3 toxin completely prevented S1P inhibition of migration. These results indicate that endogenously expressed S1P2 negatively regulates cell motility and invasion through ligand-dependent reciprocal regulation of cellular Rac and RhoA activities. We also found that in the presence of JTE013, S1P rather stimulated Rac and migration in B16 cells that overexpress either S1P1 or S1P3, unvailing counteractions between S1P2 and S1P1 or S1P3 chemotactic receptor.

0139

Epidemiologic and tumor characteristics of coexisting melanoma and nonmelanoma skin cancers

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The purpose of this study was to determine whether patients diagnosed with both melanoma and nonmelanoma skin cancers (study group) have specific demographic and clinical characteristics that differentiate them from persons with solitary melanoma (control group). The p16, bcl-2 and p53 tumor expression profiles of melanomas in each patient group were evaluated by immunohistochemistry. Our study identified several significant differences in patients diagnosed with a single melanoma in comparison to those with both melanoma and NMSCs. Patients with both tumors were significantly older (60.7 yrs vs 50.2 yrs), their melanomas were less advanced at the time of diagnosis (Breslow's depth 0.68 mm vs 1.21 mm; mean Clark's level 2.2 vs 2.8), and they were less likely to have a positive sentinel node biopsy (11% vs 29%) for lesions of similar Breslow's depth (mean depth of tumors examined 1.6 mm vs 1.8 mm). In addition, when melanomas of both groups, matched for tumor characteristics, were studied histologically for expression of p53, bcl2 and p16 protein, overexpression of p53 (indicative of p53 stabilization due to mutation) was demonstrated in a significantly higher percentage of melanomas in patients with multiple tumors (65% vs 20%). We propose that, as in patients with LMM, patients with melanoma of any histologic subtype and a previous or subsequent NMSC, are more likely to demonstrate overexpression of p53 protein, and an improved prognosis compared to their counterparts with solitary melanomas.

0140

Novel agents and targets for skin cancer chemoprevention: studies with pomegranate fruit extract

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Because primary prevention has proven inadequate in reducing rising incidence of skin cancer, there is a need to develop novel preventive approaches. Pomegranate fruit extract (PFE) derived from the tree *Punica granatum* contains several polyphenols and anthocyanidins, and its antioxidant activity, though varies depending upon the variety, is higher than that of red wine and green tea. In the present study, we evaluated whether PFE could possesses anti-tumor promoting effects. We determined the effect of topical application of PFE to CD-1 mice against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced conventional markers, and other markers of skin tumor promotion. We found that topical application of PFE (2 mg/mouse) 30 min prior to TPA (3.2 n mole/mouse) application onto the skin of CD1 mice afforded significant inhibition, in a time-dependent manner, against TPA-mediated increase in skin edema and hyperplasia. Topical application of PFE was also found to result in substantial inhibition against TPA-induced epidermal ornithine decarboxylase (ODC) activity and protein expressions of ODC and cyclooxygenase-2. Because of the role of mitogen activated protein kinases (MAPK) in inflammatory responses, proliferation, survival and apoptosis, we next assessed the effect of PFE on TPA-mediated activation of MAPK pathway in mouse epidermis. Employing Western blot analysis and immunohistochemistry, we found that topical application of PFE resulted in inhibition of TPA-induced phosphorylation of extracellular-signal-regulated protein kinases, p38 and c-Jun terminal kinases in the epidermis. Since the nuclear factor kappa B (NF-κB) has been implicated in cellular proliferation and tumor promotion, we next determined the effect of PFE on TPA-mediated activation of NF-κB pathway. Our data showed that pre-application of PFE to mouse skin significantly inhibited TPA-induced activation of NF-κB, IKKα, and degradation of IκBα. The results of our study, for the first time, provide suggestion that PFE could be a useful anti-tumor promoting agent in mouse skin.

0141

Genome-wide scanning for aberrant CpG methylations in human melanomas by methylation-sensitive-representational difference analysis

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Aberrant CpG methylations play important roles in the development and progression of a variety of human cancers. However, little is known about aberrant CpG methylations in melanomas. Here, genes that are inactivated by aberrant CpG methylations in human melanomas were searched for by a genome-wide scanning technique for aberrant methylations, methylation-sensitive-representational difference analysis (MS-RDA) [PNAS, 94:2284, 1997]. Eighty DNA fragments that were differentially methylated between the primary human epidermal melanocyte, HEM, and a melanoma cell line, MeWo, were isolated by MS-RDA using two methylation-sensitive restriction enzymes, *HpaII* and *SacII*. Seventy-one DNA fragments differentially methylated between HEM and another melanoma cell line, WM-266-4, were similarly isolated. A total of 144 DNA fragments were found to be aberrantly methylated in MeWo and/or WM-266-4, but not in HEM. By analyzing their flanking genomic structures using a human genome database, 107 of the 144 DNA fragments were found to be derived from CpG islands (CGIs), and 16 of them were located in 5′ regions of genes. Methylation statuses of these CGIs were analyzed by methylation-specific PCR using six melanoma cell lines, including MeWo and WM-266-4, and HEM. Six of the 16 CGIs were shown to be aberrantly methylated in some of the melanoma cell lines but not in HEM. The six genes downstream of these aberrantly methylated CGIs are expected to be silenced, and are candidate tumor-suppressor genes in melanomas.

0142

Oleandrin: a novel agent for skin cancer chemoprevention

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Primary prevention has proven inadequate in reducing rising incidence of skin cancer, emphasizing the need to develop novel skin cancer chemopreventive strategies. Oleandrin, derived from the leaves of *Nerium oleander* has been shown to possess anti-inflammatory and tumor cell growth-inhibitory effects. Here, we provide evidence that oleandrin could possesses anti-tumor promoting effects. We determined the effect of topical application of oleandrin to CD-1 mice against 12-O-tetradecanoylphorbol-13-acetate (TPA), a widely studied skin tumor promoter, -induced conventional and novel markers of skin tumor promotion. Topical application of oleandrin (2 mg/mouse) 30 min prior to TPA (3.2 n mole/mouse) application onto the skin afforded significant inhibition, in a time-dependent manner, against TPA-mediated increase in cutaneous edema and hyperplasia, and epidermal ornithine decarboxylase activity and protein expression. In search for novel markers of skin tumor promotion, we found that TPA application to mouse skin resulted, as an early event, in an increased expression of phosphatidyinositol 3-kinase (PI3K), phosphorylation of AKT at threonine^{308}, and activation of nuclear factor kappa B (NF-κB). Topical application of oleandrin prior to TPA application to mouse skin resulted in significant reduction in TPA-induced expression of PI3K and phosphorylation of AKT, and inhibition of NF-κB activation. NF-κB is a eukaryotic transcription factor that is critically involved in regulating the expression of various genes that participates in inflammation, apoptosis and cell proliferation. Employing Western blot analysis and immunohistochemistry, we found that oleandrin application to mouse skin resulted in inhibition of TPA-induced activation of NF-κB, IKKα, and degradation of IκBα in mouse epidermis. Our data suggest that oleandrin could be a useful anti-tumor promoting agent because it inhibits several biomarkers of TPA-induced tumor promotion in an in vivo animal model. One might envision the use of chemopreventive agents such as oleandrin in an emollient or patch for chemoprevention or treatment of skin cancer.

0143

Pigment epithelium-derived factor, an inhibitor of angiogenesis, suppresses melanoma growth by inducing tumor cell apoptosis

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Tumor angiogenesis is essential for the growth of primary and metastatic tumors. It is regulated by proangiogenic factors and also anti-angiogenic factors secreted by tumor and host cells. Pigment epithelium derived factor (PEDF), recently identified as one of the inhibitors of angiogenesis, is known to induce endothelial cell apoptosis. Our aim was to elucidate the mechanism of melanoma grow inhibition by PEDF. First, we determined the expression levels of PEDF in human malignant melanoma cell. PEDF was shown to be constitutively produced by several melanoma cell lines. We transfected the human PEDF gene into melanoma cell (G361). Stable PEDF transfectant cells (PEDF-G361) secreted high level of PEDF compared with that of Mock-transfectant cells (Mock-G361). The medium obtained from PEDF-G361 culture, induced higher levels of endothelial cell apoptosis compared with that of Mock-G361 culture medium. Interestingly, the PEDF-G361 proliferation rate was significantly lower than Mock-G361. Blocking the Fas receptor using anti-Fas Ab increased the proliferation rate, indicating PEDF mediated melanoma cell apoptosis was dependent on the Fas pathway. Furthermore, the tumor growth rate of PEDF-G361 xenografts in athymic mice was reduced compared to Mock-G361. In conclusion, PEDF mediates tumor inhibition not only by its anti-angiogensis effect but also by direct induction of tumor apoptosis.

0144

Down-regulation of μ opioid receptor in Fas/FasL knock out mice

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Opioids such as morphine can promote programmed cell death by enhancing the expression of pro-apoptotic Fas receptor protein; This effect goes through the sustained activation of opioid receptors. In different tissues such as neural crest, endometrium and lymphocytes opioids control apoptosis. Additionally opioid receptors induce apoptosis in lung cancer cells. We have published that functional μ opiate receptor exist in human keratinocytes and β endorphin between 10-100 nM down regulate μ opiate receptor expression. In this work we show that β endorphin at 60 nM induces apoptosis in epidermis of cultured skin equivalent (appligraf) using tunnel reaction. In addition We also observe the downregulation of μ opiate receptor in basal cell carcinoma cells in which the Fas receptor system is downregulated. This points to the intercorrelation of Fas receptor with μ opiate receptor. Therefore, we investigate the μ opiate receptor expression in epidermis of Fas receptor deficient (lpr) mice and Fas ligand deficient (gld) mice. Indeed, our immunohistochemstry results indicate that there is downregulation of μopiate receptor in both lpr and gld mice; The downregulation of μ opiate receptor in lpr mice are more dramatic than in gld mice. These data show that opioid receptor system is involved in apoptosis in epidermis and suggest a connection between opioid receptors and Fas receptor system in epidermis. This could be important in control of UV induced skin cancers, especially basal cell carcinoma because apoptosis plays an important role in the elimination of irreversibly UV damaged keratinocytes.

0145

Tumor development and gene expression profiling following epidermal abrasion

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The v-Ha-ras transgenic mouse (Tg.AC) develops papillomas following several tumor promotion regimens, including chemical treatment and UV radiation, and much of our evidence indicates a follicular origin of tumors in these mice. To directly test this hypothesis, we subjected Tg.AC mice to a single epidermal abrasion. 100% of the abraded mice developed papillomas by 5 weeks post-abrasion, compared to 0% in the non-abraded controls. Transgene expression was detected by 18 days post-abrasion by RT-PCR and in situ hybridization, with expression localizing to the proliferative (PCNA-positive) compartment of the lesions. High density microarray analysis was conducted on RNA from singly abraded Tg.AC and FVB/N (background strain) mice at days 5, 9, and 18 post-abrasion to: 1) generate an expression profile during epidermal remodeling, and 2) to elucidate genes or gene pathways uniquely affected by ras transgene expression. The abraded skin of both strains of mice highly expressed genes in the Epidermal Differentiation Complex (ECD) over all three-time points, including S100A8 (calgranulin) and sprr2A. In addition, a serine protease inhibitor, Stefin 3, was highly overexpressed, highlighting along with the ECD genes the extensive terminal differentiation and stratification processes occurring with remodeling. Genes discriminating Tg.AC from FVB/N at the various time points fell into several categories, including extracellular matrix, DNA excision repair, and inflammation. In summary, we have demonstrated that a single epidermal abrasion is sufficient to induce papillomas in the Tg.AC mouse, implicating hair follicle stem cells in tumor formation. In addition, high density microarray analysis has provided insight into gene expression changes occurring over time during epidermal remodeling.

0146

DNA methylation profile of malignant melanoma, basal cell carcinoma, and squamous cell carcinoma of the skin

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Epigenetic regulation of the expression of tumor suppressor genes plays important role in tumor development. DNA methylation of gene promoter is a major pathway of the epigenetic regulation, however, its detail has not well studied in skin neoplasms. With the power of non-isotopic cytosine extension assay and methylation specific PCR, we analyzed promoter methylation status of p16, p14, p15, e-cadherin, and von Hippel-Lindau tumor suppressor genes in various skin cancers. In squamous cell carcinoma gene promoter methylation of each gene was detected in 8/12, 2/12, 0/12, 12/12, and 0/12 cases, respectively. In basal cell carcinoma and malignant melanoma p16 and e-cadherin promoter were methylated in less than half the cases; p14, p15, or VHL genes were not methylated. Promoter DNA methylation may play an important role in the development of skin neoplasms, especially in squamous cell carcinoma.

0147

CXCR4 activates beta1 integrin-mediated binding of B16 murine melanoma cells to lung-derived endothelial cells

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The chemokine receptor, CXCR4, is expressed by human melanomas and its ligand, CXCL12, is frequently produced at sites of melanoma metastasis. We previously showed that CXCR4-transduced B16 cells (CXCR4-B16) metastasized 6-10 fold better than control B16 cells to the lung after i.v. inoculation and bound 3-fold better (vs. control cells) to pulmonary endothelial cells (EC). Herein, we examine CXCR4-mediated binding of B16 to EC and recombinant adhesion molecules in vitro in order to determine the role of tumor- and EC-derived adhesion molecules in tumor metastasis. First, by flow cytometry, unstimulated primary lung EC showed constitutive expression of VCAM-1 whereas skin-derived EC did not. All B16 cell lines tested showed constitutive expression of beta1 (CD29), but not beta2, integrins. Second, under physiologic shear stress conditions (1.5 dynes/cm2) in a flow chamber, CXCR4-B16 cells bound to monolayers of lung EC at least two-fold more efficiently than to skin-derived EC ($p=0.002$). As recorded by real-time video, CXCR4-B16 arrest on EC was rapid, resistant to further shear, and showed no evidence of transient rolling. Interestingly, CXCR4-B16 cell binding to EC was completely blocked by anti-CD29 mAb. Under similar flow conditions, CXCR4-B16 cells arrested 40-fold better than mock-transduced-B16 cells on plastic plates coated with recombinant VCAM-1-Ig and CXCL12 ($p<0.0001$). Third, CXCR4-B16 exposed to CXCL12 rapidly increased binding affinity (within 30 sec) for soluble VCAM-1-Ig as detected by a flow cytometric assay. Thus, using a novel method for observing tumor cell-EC interactions, we show that beta1-integrin plays a critical role in CXCR4-mediated binding of B16 tumor cells to both EC and recombinant VCAM-1. Consequently, the beta 1 integrin may be a potential target for inhibition of tumor metastasis. Lastly, the differential expression of VCAM-1 by lung vs. skin-derived EC may explain why the lung is a favored site of initial tumor localization following vascular dissemination.

0148

Topical tacrolimus potentiates carcinogenesis in mouse skin and reduces the lymph node CD4/CD8 ratio

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Tacrolimus (FK506) is a potent macrolide immunosuppressant, widely used for preventing graft rejection in liver and kidney transplantation. Recently, topical FK506 has been used to treat atopic dermatitis (AD) because of its T cell immunosuppressive effect. However, since 7% of patients undergoing systemic administration of FK506 for liver transplantation subsequently develop malignancy (Transplantation, 66, 1193, 1998), we sought to investigate the carcinogenic effect of topical FK506. We used a standard cutaneous tumor induction protocol in which the shaved dorsal skin of CD-1 mice was treated with the tumor initiator of DMBA, followed by promoting treatment with TPA, with or without FK506 ointment. After 14 wk, there was marked synergy between FK506 and the DMBA/TPA regimen (new tumors per mouse per week in the FK506/DMBA/TPA group vs. DMBA/TPA group, $p<0.01$). The CD4/CD8 ratio in axillary and inguinal lymph nodes in FK506-treated mice was significantly decreased. The CD4/CD8 ratio in blood was also reduced by 5 wk after initiation, even though the serum FK506 concentration after topical application is 50 to 100 fold lower than when the drug is systemically administered. The concentration of FK506 in lymph nodes after topical treatment was found not to differ significantly from that after systemic FK506 administration. These findings suggest that topical application produces high concentrations of FK506 in lymph, with subsequent depletion of CD4 T cells. Tumor formation was also accelerated in mice treated with topical FK506 after UVL irradiation, compared with control mice irradiated with UVL alone. Use of topical FK506 for the treatment of AD has become widespread, largely because of its steroid-sparing effect. Our data suggest that restraint should be exercised in prescribing this regimen, especially for use on the face, and the safety of this drug should be re-examined.

0149

Survivin targeting triggers rapid translocation of mitochondrial apoptosis-inducing factor in melanoma cells

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Apoptosis, or programmed cell death, is commonly dysregulated in melanoma and resistance to apoptosis likely accounts for treatment failure in patients with advanced disease. We have shown previously that the Inhibitor of Apoptosis protein Survivin, a known caspase inhibitor, is highly expressed in melanoma and that blocking Survivin causes melanoma cell apoptosis and prevents tumor formation. To investigate how Survivin protects melanoma cells from apoptosis, we examined the kinetics of apoptotic events using melanoma cells stably transfected with a tetracycline-regulated Survivin dominant-negative mutant (Survivin-T34A). Late-stage apoptosis (DNA fragmentation) occurred in these cells by 24-48 hrs, but not in control cells transfected with wild-type Survivin. Apoptosis induced by Survivin targeting, but not UVB, was resistant to the pan-caspase inhibitor z-VAD-fmk. Immediately following expression of Survivin-T34A (Western blot, 4 hrs), we detected phosphatidylserine transposition (Annexin V binding, 4 hrs), mitochondrial depolarization (JC-1 shift, flow cytometry, 11 hrs), and mitochondrial release of cytochrome c and Smac/DIABLO (Western blot, 8 hrs). These mitochondrial events were preceded by nuclear translocation of mitochondrial apoptosis-inducing factor (AIF, Western blot and immunofluorescence, 4 hrs), known to cause mitochondrial content release and mediate caspase-independent apoptosis. By contrast, activation of terminal caspase-3 (48 hrs) and upstream caspase-8 (48 hrs) and caspase-9 (24-48 hrs), as assessed by precursor loss and appearance of cleavage fragments on Western blots, was incomplete and relatively delayed. Specific inhibitors of caspase-8 (z-IETD-fmk) and caspase-9 (z-LEHD-fmk) were less protective than z-VAD-fmk in preventing apoptosis induced by Survivin targeting. Thus despite its reported function as a caspase inhibitor, Survivin primarily acts to restrain AIF in mitochondria and thereby protect melanoma cells from caspase-independent apoptosis.

0150

Pericyte-like location of GFP melanoma cells: *ex vivo* and *in vivo* sudies of extravascular migratory metastasis

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We have previously described in melanoma the angio-tumoral complex, in which tumor cells occupy a pericyte-like location on the outside of the endothelium. The pericyte-like location of melanoma cells raises the possibility that tumor cells are migrating along the abluminal surface of the endothelium, a mechanism termed Extravascular Migratory Metastasis. In this study, we have used human and murine melanoma cells that stably express Green Fluorescent Protein to follow the interaction of tumor cells with vessels over time. We have developed ex vivo model systems to study melanoma cell interactions with vessels that have sprouted from rat and chick aortic rings. We observed tropism between tumor cells and endothelial cells, and a pericyte-like location of tumor cells spreading along the vascular channels. We have also used the in vivo chick chorioallantoic membrane assay. Using fluorescence microscopy, we have observed the dissemination pathway of melanoma cells along vessels at the single cell level. Single tumor cells were regularly observed spreading along vessels several centimeters from the tumor. Confocal laser scanning microscopy and histopathology confirmed the pericyte-like location of tumor cells, without any observation of mosaic vessels or intravasation of tumor cells. Despite the fact that C8161 cells growing alone in Matrigel were able to form connected structures, we did not observe vascular mimicry in the ex vivo and in vivo assays.

0151

Tazarotene-induced gene-3 is suppressed in basal cell carcinomas and up-regulated by tazarotene *in vivo*

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Tazarotene, a synthetic RAR-β/γ selective retinoid, is approved for acne and psoriasis. Tazarotene-induced genes identified by differntial display PCR from human keratinocytes include Tazarotene-induced gene 3 (TIG-3), an RAR-β/γ selective retinoid-induced class II tumor suppressor that inhibits cell growth [*Disepio et al,PNAS,1998*]. TIG-3 is downregulated in both psoriasis and squamous cell carcinomas [*Duvic et al,CCR,2000*]. To study TIG-3 in basal cell carcinomas (BCC) and as chemoprevention, we enrolled 22 patients in a clinical trial to measure TIG-3 protein and mRNA in baseline and treated BCC and paired normal skin. Immunohistochemistry with anti-rTIG-$3_{1\text{-}164}$ and *in situ* hybridization with riboprobes were conducted in 19 pairs of BCCs from 14 patients before and after 0.1% Tazarotene gel applied for 6-12 weeks before surgery. Loss of or decreased TIG-3 protein was detected in 14 of 19 BCC (74%) and mean level was 2.17 fold less in BCC than in overlying normal epidermis (P=0.00002). Decreased TIG-3 mRNA was detected in 18 of 19 lesions (95%) and mean level was 2.63 fold less than in overlying epidermis (P=0.00001). Eleven of 16 BCC(69%) evaluated after therapy had increased TIG-3 protein expression of 1.96 fold compared to baseline (P=0.0002). Similarly, ten of 16 tumors (63%) had increased TIG-3 mRNA, with 2.67 fold increase after therapy (P=0.0276). Twelve of 16 (75%) BCC with decreased TIG-3 mRNA at baseline had clinical responses and 9 of 12 showed upregulation of TIG-3 mRNA. Expression of TIG-3 protein and mRNA is selectively decreased in BCCs and is restored by treatment with topical tazarotene in patients. TIG-3, as a retinoid inducible tumor suppressor, may explain retinoid activity in skin cancer, and serve as an intermediate biomarker for chemoprevention trials.

0152

Resistance of CD1-deficient mice to UV skin carcinogenesis is associated with increased keratinocyte apoptosis

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CD1 is a nonpolymorphic major histocompatibility complex I-like molecule. Murine CD1 has been implicated in the development and function of Natural Killer T cells. Recent studies have demonstrated that CD1-/- mice are resistant to UV-induced immune suppression and skin damage compared to wild-type mice. Our recent studies have shown that CD1-/- mice are also more resistant to skin cancer induced by solar-simulated UV radiation than wild-type mice. To determine the mechanisms involved in the resistance of CD1-/- mice to UV-induced skin damage and carcinogenesis, we investigated the time course and kinetics of keratinocyte cell death after acute and chronic UV irradiation with a solar simulator. After acute UV exposure (5 kJ/m2), the number of TUNEL-positive keratinocytes peaked at 24-48 hours after irradiation in both groups. However, the TUNEL-positive keratinocytes were eliminated from the skin at 72 hours post-UV in wild-type mice, but they still persisted until 96 hours post-UV in CD1-/- mice. The kinetics of p53 protein expression closely followed the kinetics of apoptotic cell death. The persistence of apoptotic keratinocytes in CD1-/- mouse skin correlated with high levels of p53 expression at 96 hours post-UV. Chronic UV irradiation (5 kJ/m2, 3x/wk) for 1-3 weeks resulted in induction of a significantly higher number of apoptotic keratinocytes in CD1-/- mice than in wild-type mice. After 12 weeks of chronic UV, clusters of p53-positive, abnormal keratinocytes were present in wild-type mouse skin, but not in CD1-/- mouse skin. In addition, after 30 weeks of chronic UV, clusters of p53-positive, abnormal fibroblasts were present in wild-type mouse skin, but not in CD1-/- mouse skin. These results indicate that the resistance of CD1-/- mice to UV-induced skin damage and UV carcinogenesis may in part be due to increased cell death and elimination of keratinocytes and fibroblasts containing DNA damage and p53 mutations.

0153

Apoptotic resistance by AAA-protein, SPAF in malignant melanoma

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Malignant melanoma is one of the most formidable diseases arising from skin. This disease is resistant to apoptosis and refractory to conventional therapeutic regimens. Apoptosis can be induced through various pathways, implying a variety of mechanisms by which malignant melanoma cells may develop apoptotic resistance. In search of altered gene expression in a cloned epidermal model of carcinogenesis, we have identified a novel spermatogenesis associated factor (SPAF) that is altered and overexpressed in an aggressive squamous cell carcinoma. SPAF protein contains two ATPase modules which classify it as a novel member of AAA-protein family (ATPase associated with diverse activities). Analysis of SPAF mRNA expression in various human cancers revealed that SPAF is highly expressed in the majority of malignant melanoma samples tested. Furthermore, analysis of the human SPAF gene unveiled a unique bi-directional genomic structure in connection with FGF-2, a growth factor overexpressed in virtually all malignant melanoma. The prevalent expression of SPAF and FGF-2 in malignant melanoma and their genomic connection suggest that SPAF expression is an oncogenic event that may synergize with FGF-2 in melanomagenesis. In normal testis, SPAF expression is restricted to spermatogonia in the early stage of spermatogenesis. However SPAF expression is exclusively absent in spermatogonia undergoing apoptosis. In view of its localization to mitochondria and membership in a AAA subfamily involving membrane fusion, SPAF is postulated to increase the apoptotic threshold by stablizing the mitochondrial membrane. Ongoing studies are, 1) to evaluate the effect of SPAF expression on apoptotic resistance in malignant melanoma cells by means of RNA interference, and 2) to detect SPAF expression in radial growing versus vertical growing malignant melanoma. These studies will not only shed light on SPAF activities as a novel mechanism in apoptosis and melanomagenesis, but also may provide targets for molecular prognosis and therapy for malignant melanoma.

0154

Shift of gap junctional communication and cell adhesion during melanoma development

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There is a dynamic shift in cell adhesion molecule expression during melanoma development - namely the down-regulation of E-cadherin and up-regulation of N-cadherin. The loss of E-cadherin in malignant melanoma (MM) is associated with gap junction incompatibility with keratinocytes and the gain of N-cadherin with development of gap junctions (GJ) with fibroblasts and endothelial cells. Cell adhesion by cadherins might be required for the formation of GJ. GJ play a major role in cell-cell-communication. They form channels between adjacent cells allowing the diffusion of small molecules (e.g. second messengers). GJ consist of two hemichannels called connexons, each formed by six transmembrane proteins called connexins (Cx). Ten of the known 21 connexins are expressed in human skin. GJ formed by different connexins are selective for different molecules. Thereby they control the specificity of cell-cell-communication. This work concerns the expression of Cx30 and Cx26 in MM and melanocytic nevi and in epidermis adjacent to these lesions. Investigations were performed by immunofluorescence microscopy using specific antibodies. Cx30 and Cx26 are only weakly, if at all, expressed in normal interfollicular epidermis. Now we show that both proteins neither occur in MM nor in melanocytic nevi. In contrast both Cx30 and Cx26 are expressed in the epidermis adjacent to MM but not to melanocytic nevi. These results clearly show an induction of Cx30 and Cx26 in normal interfollicular epidermis adjacent to MM. There is no such induction in benign melanocytic nevi. Previously we demonstrated similar results investigating GJ proteins in malignant epithelial skin tumours and Merkel Cell carcinoma. There was no such induction in the epidermis adjacent to semimalignant and benign lesions. So one might suggest that the change of GJ composition in the epidermis adjacent to malignant skin tumours may play a role in the metastasation of these tumours.

0155

Characterisation of the cylindroma tumour suppressor gene

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Familial and sporadic cylindromas, benign adnexal skin tumors, are caused by mutations in the cylindromatosis (CYLD) gene located on chromosome 16q12. The ubiquitously expressed CYLD gene encodes a putative tumor suppressor molecule. Protein motif analysis revealed a central domain containing CAP-Gly sites found in microtubule-binding (MT) proteins, and a C-terminal region with similarities to the active site of deubiquitinating enzymes and to Zn fingers. Transfection of an expression vector with the FLAG-tagged full-length CYLD cDNA (pCYLD1) into 293T and cos7 cells demonstrated that CYLD is localized to the perinuclear compartment. Confocal immunofluorescence microscopy and spin-down microtubule binding assays revealed no association between CYLD protein and microtubules. To examine the biological role of CYLD, its effect on cell growth was investigated by colony transformation assays in 293T cells using cotransfection with pCYLD1 and pBabe-puro. After 2-week culture under puromycin selection the number of resistant colonies was drastically reduced in CYLD transfected cells compared with pBabe-puro alone, arguing in favor of a strong cell growth inhibitory effect of CYLD. Furthermore, colony transformation assays using truncated forms of CYLD indicated that the N-terminal domain alone strongly suppresses cell growth. A yeast two-hybrid screen using HaCaT cDNA library as prey and full-length CYLD as bait, and coimmunoprecipitation in 293T cells, identified 2 novel proteins, p30 and p40, which physically associate with CYLD. Furthermore, alpha-galactosidase assays in yeasts demonstrated that it is the CAP-Gly domains of CYLD which specifically interact with p30. In situ hybridisation and immunostaining showed that patched1 and Gli2 are overexpressed in cylindroma. These data indicate that full-length CYLD does not bind to microtubules, the N-terminal domain of CYLD has a strong suppressive activity on cell proliferation and that the Shh signalling pathway is activated in cylindromas.

0156

Characterization of basal cell carcinoma by multifunctional optical coherence tomography

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The puropse of our study is to describe features of basal cell carcinoma (BCC) identified using multifunctional optical coherence tomography (OCT). Diagnosis of BCC by biopsy is invasive, painful, and the information is available at only one time point. Moreover, it is subject to sampling error, including misidentification of aggressive sub-types. As more BCCs are treated with non-surgical intervention, there is a need to monitor these lesions for completeness of resolution and for early detection of recurrence. OCT uses broad-band light to harmelssly generate non-invasive vertical "optical biopsy" images of the skin. It has the potential to become a useful tool for BCC management, but it has not yet been applied to the analysis of skin cacer. OCT has been available for several years, but no suitable system has been developed for skin cancer imaging. We developed a device with several enhancements, including improved image quality as well as simultaneous calculation and display of Doppler analysis (for blood flow measurenents), polatization sensitivity (to detect subtle changes in conneective tissue structure), and real-time imaging (to render the device practical for the clinician). Multifunctional OCT was used to identify features of BCC which are distinguishible from normal perilesional skin. Imaging of BCCs reveal several tumor characteristics identified by multifunctional OCT. Altered intensity signals of the tumor lobules, septae, and tumor stroma are clearly visualized; Doppler information detects tumor-associated neovasculatization; the polarization states of tumor components are altered when compaed with the pattern normally observed in the papillary and reticular dermis; the device is practical for clinical use because it is fiber-optic based and the information is presented in real-time. Multifunctional OCT has the potential to become a useful and practical clinical aid for BCC diagnosis and monitoring of therapy.

0157

Alpha-melanocyte stimulating hormone alters the apoptotic response to ultraviolet radiation

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Variants of the melanocortin 1 receptor (*MC1R*) gene are associated with red hair and fair skin type in humans, and increase susceptibility to skin cancer. Alpha-melanocyte stimulating hormone (αMSH) has potent biological activity in addition to its effects on pigmentation (e.g. anti-inflammatory and immunomodulatory), and recent research by several groups suggests that *MC1R* variants may increase the risk of developing skin cancer through their effects on non-pigmentary pathways. Exposure to ultraviolet radiation (UVR) causes skin cancer, and therefore it is possible that αMSH / MC1R signalling may alter the non-pigmentary response of cells to UVR, for example through effects on cell viability. In this study, we have examined the effects of αMSH on UV-induced apoptosis, independent of the protection afforded by melanin pigmentation, in *MC1R/Mc1r* null amelanotic melanoma cells stably transfected with wild type human *MC1R*. Transfected B16G4F murine and RPMI-7951 human melanoma cells were cultured in the presence or absence of 10^{-6}M αMSH prior to and following UV irradiation with a TL12 lamp. Cells were analysed for apoptosis using flow cytometry for annexin V cell surface binding at 24 hours following UV-exposure. All experiments were carried out in triplicate wells, and on three separate occasions. Sensitivity to UV-induced apoptosis varied between the two *MC1R* transfected melanoma cell lines. However, αMSH significantly increased the percentage of cells binding annexin V; B16G4F mean 17.8 ± SEM 6.3, B16G4F+αMSH mean 22.7 ± SEM 6.9 (p=0.020), and RPMI-7951 mean 29.9 ± SEM 7.2, RPMI-7951+αMSH mean 36.5 ± SEM 9.2 (p=0.028). The results suggest that signalling via the αMSH / MC1R pathway increases apoptosis following exposure to UV. As this may have implications for skin cancer susceptibility, further work using variant *MC1R* stable transfectants is investigating the effects of *MC1R* variants on this parameter.

0158

Gene expression profile in squamous cell carcinoma complicating recessive dystrophic epidermolysis bullosa using cDNA microarray analysis

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Recessive dystrophic epidermolysis bullosa (RDEB) sufferers have an increased risk of developing squamous cell carcinoma (SCC) with more than 75% developing SCC by the age of 60-years. It is the major cause of premature death in this disorder. However, little is known about why these tumors develop and why they behave so aggressively. To explore tumor biology further, we compared the gene expression profiles in SCCs and uninvolved skin from RDEB patients (n=3) with SCCs and normal skin from non-EB patients (n=3). Total RNA was extracted from 6mm punch biopsies. cDNA was radiolabeled with ^{33}P and hybridized to Dermarray™ nylon filters (Research Genetics Inc.) that contain 4022 skin-expressed genes. Images were scanned using a Storm 840 PhosphorImager and analysis was performed with Pathways™ 4 software. Overall, 58 genes in the RDEB group and 68 genes in the non-RDEB group showed differential gene expression, defined as at least a two-fold difference between lesional and non-lesional tissue. Selected genes were confirmed by RT-PCR and/or immunohistochemistry. Both groups showed down-regulation of epidermal differentiation complex genes in SCC including profilaggrin, keratin 10 and keratin 1. Other down-regulated genes in both groups included death-associated protein kinase 3 (ZIP kinase), lysyl oxidase and D-aspartate. Matrix metalloproteinase 1 was up-regulated in SCC of all non-RDEB subjects and 2/3 SCC of the RDEB group. Differences in gene expression between the two groups also included laminin 5, which was up-regulated in the non-EB group but not in the EB group. Tumour rejection antigen gp96 (ZAP128) was down-regulated in all of the non-RDEB group but none of the RDEB group. Insulin like growth factor binding protein 3 was more up-regulated in the non-EB SCCs than EB SCCs This study highlights differences in the gene expression profile of SCC complicating RDEB and provides new insight into tumor biology in this genodermatosis.

0159

Reactive oxygen is implicated in Epstein-Barr virus-induced neoplasia

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Epstein-Barr Virus (EBV) is the virus implicated in the vast majority of virally-induced human cancers. Among the cancers implicated in EBV-induced neoplasia are cutaneous leiomyosarcoma, Burkitts lymphoma, Hodgkins disease, nasopharyngeal carcinoma, and gastric carcinoma. The precise mechanisms of EBV-induced oncogenesis are not completely understood. We have previously demonstrated that hypermethylation of tumor suppressor genes is a hallmark of reactive oxygen species (ROS) induced carcinogenesis, and other laboratories have demonstrated that EBV-induced cancers display hypermethylation of the p16ink4a tumor suppressor genes. We have also demonstrated that reactive oxygen induces the angiogenic switch. We analyzed a series of EBV-induced and sporadic (EBV-) Burkitts lymphoma cell lines for reactive oxygen. EBV-induced lymphomas demonstrated high levels of ROS compared with EBV- Burkitts lymphoma cells. Infection of EBV negative cells with EBV resulted in increased levels of ROS. In addition, EBV+ cells demonstrated activation of MAP kinase, while EBV- cells did not show activation of MAP kinase. Treatment of EBV positive cells with a potent antoxidant, ebselen, led to decreased growth and inhibition of NFkB signaling. NFkB signaling in EBV negative cells is dependent upon PI3 kinase signaling, but not reactive oxygen. Pharmacologic therapy with appropriate antioxidants may lead to prevention of EBV-induced neoplasia in immunocompromised patients, and to enhanced therapies for patients with existing tumors.

0160

GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter

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SHh binds to its receptor patched (Ptch), leading to the activation and repression of target genes via zinc-finger Gli family transcription factors. These proteins act in a combinatorial fashion: GLI1 and GLI2 have activating effects, whereas GLI3 (and at times GLI2) have repressor activities. Deregulation of the SHh pathway leads to a number of human diseases, including birth defects and basal cell carcinoma (BCC) of the skin. Hereditary as well as sporadic BCCs are associated with PATCHED mutations. In transgenic mouse model systems targeted expression of GLI1 or GLI2 induces BCC formation. However, our understanding of the molecular mechanisms of SHh signalling and GLI activity in human keratinocytes and their role in tumourigenesis is still very limited. We have previously shown that GLI1 is expressed in the outer root sheath (ORS) of hair follicles and in BCC but not in normal interfollicular epidermis, and that GLI2 is expressed at elevated levels in BCC compared to normal skin. We also identified a positive feedback mechanism between GLI1 and GLI2 in the HaCat cell line using time course gene expression analysis indicating that GLI1 is likely to be directly regulated by GLI2. In this study we have shown with western blot and RT-PCR analysis that retrovirally expressed GLI2 induces endogenous GLI1 expression in human primary keratinocytes. In-addition, band shift assays show that GLI2 specifically binds the GLI binding consensus sequence in the GLI1 promoter confirming that GLI2 directly activates GLI1. Finally, using in situ hybridisation we have shown that GLI2 is expressed in interfollicular epidermis and ORS of hair follicles in normal skin as well as in BCC tumour islands. These results suggest an important role for GLI2 in regulating epidermal proliferation and skin tumorigenesis.

0161

(-)-Epigallocatechin-3-gallate directly induces apoptosis and inhibits proliferation of human melanoma cell lines

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Melanoma accounts for ~ 4% of all skin cancer cases and ~79% of skin cancer-related deaths in the USA. Because of the growing incidence and high mortality of melanoma, novel approaches for its management are warranted. Chemoprevention by naturally occurring agents present in the diet may be a useful strategy. Green tea polyphenolic compounds have shown great promise as chemopreventive agents in a variety of cell lines and animal tumor model systems. EGCG, the major polyphenolic antioxidant present in green tea, is believed to be critical for its chemopreventive effects. Employing a series of human melanoma cell lines A-375 and G-361 (malignant melanoma) and Hs-294T (metastatic melanoma), and normal human epidermal melanocytes (NHEM), we evaluated the chemopreventive effects of EGCG against cells of melanocytic lineage. EGCG treatment was found to result in a dose-dependent decrease in cell viability by trypan blue exclusion. The MTT assay revealed that EGCG (1, 2, 5, 10, 20, 40, 80 µg/ml) treatment of the cells resulted in significant dose-dependent inhibition in the proliferation of all melanoma cell lines studied. This effect was significantly greater than that in NHEM at similar EGCG dose. Western blot analysis revealed that EGCG- treated melanoma cell lines showed significant evidence of apoptosis as evident from the cleavage of 115 kDa poly (ADP-ribose) polymerase protein (PARP) into ~89 kDa and ~27 kDa proteins. In addition, EGCG treatment of the melanoma cell lines resulted in a significant i) decrease in the expression of Ki-67 nuclear protein (by immunostaining of cytospin preparations) and the colony formation ability (by soft agar colony formation assay), and ii) increased binding to Annexin-V (by flow cytometry). To our knowledge, this study is the first to demonstrate the direct, selective anti-proliferative/pro-apoptotic effects of EGCG against melanoma cell lines. This raises the possibility that EGCG, at physiologically attainable concentrations, may have chemopreventive and/or therapeutic potential for human melanoma.

0162

Enhanced nodal metastasis and protection from Fas-mediated apoptosis in B16 murine melanoma transduced with CCR10

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CCR10 is a CC chemokine receptor that has been reported to be commonly expressed (at the mRNA level) by human melanoma cells. One of its known ligands, CCL27, is constitutively expressed by epidermal keratinocytes. Using specific anti-CCR10 antibodies, we have determined that CCR10 is expressed by human melanoma. To evaluate the potential role of CCR10 in melanoma progression, B16 murine melanoma cells were retrovirally transduced with cDNA for CCR10 (CCR10-B16) and luciferase (for quantitative analysis). Following footpad inoculation ($4x10^5$ cells/footpad), CCR10-B16 cells formed tumors that were 40% larger than those derived from vector-transduced-B16 cells ($p<0.005$). Regional (popliteal) LN metastasis (>90%) was often observed with CCR10-B16-injected mice while nodal metastasis was uncommon (<20%) in pLNCX2-B16-injected mice. After ear inoculation ($1x10^5$ cells/ear), 13 of 15 mice injected with CCR10-B16 cells developed a solid tumor with frank metastases to the cervical lymph node (LN) occurring in 14 of 15 mice. In contrast, mice injected with vector (pLNCX2)-transduced B16 cells rarely formed tumors in the ear (2 of 15) or showed metastasis to the draining LN (3 of 15). Histologically, a dense dermal inflammatory cell infiltrate was observed in the ear skin of pLNCX2-B16-injected mice at the inoculation site, but not CCR10-B16-injected mice. To address whether CCR10 ligation might protect tumor cells from Fas-mediated apoptosis (a major mechanism of host tumor defense), CCR10-B16 cells were treated with interferon-gamma to upregulate Fas expression, exposed to FasL (+/-CCL27) to induce apoptosis, and then stained with annexin V-FITC to quantify apoptosis. In the presence of CCL27, apoptosis was reduced from 52% to 22% in CCR10-B16 cells ($p=0.009$, baseline apoptosis=17%). Thus, CCR10 plays a potentially critical role in the growth, survival, and metastasis of melanoma cells and may provide a mechanism for tumor cells to evade host immune destruction.

0163

Effective DEAE dextran-mediated transfection of primary blood lymphocytes, dendritic cells, and immature hematopoietic cells for use in functional DNA repair assays

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The host cell reactivation (HCR) assay measures the ability of transfected cells to repair plasmid DNA damage as reflected in the recovery of luciferase activity. We established the HCR assay to measure DNA repair capacity of primary blood lymphocytes, dendritic cells, and CD34+ cells using an optimized DEAE dextran transfection protocol. $2x10^5$ cells were transfected with 250 ng plasmid DNA. Primary blood lymphocytes were isolated by Ficoll gradient and, after cryopreservation, PHA stimulated for 3 days. Immature dendritic cells were derived from primary blood monocytes (6 day cultures in the presence of IL-4 and GM-CSF). CD34+ cells were isolated from cord blood by MACS and, after cryopreservation, cultured in stem cell medium. We obtained luciferase activities 1000-fold, 200-fold, and 350-fold above background activity with undamaged plasmids in the different cell types, respectively. In addition, we established a plasmid shuttle vector mutagenesis assay using primary blood lymphocytes. $2x10^6$ PHA stimulated lymphocytes were transfected with 250 ng reporter plasmid using DEAE dextran. After 2 days the plasmids were harvested from the cells and transformed into bacteria. Blue colonies indicate complete DNA damage repair whereas white bacterial colonies indicate mutations resulting from unrepaired DNA damage. These two functional DNA repair assays established with different primary cells will enable us to test individuals for their capacity of different DNA repair pathways like nucleotideexcision, baseexcision, or double strand break repair depending on the type of DNA damage induced in the plasmid.

0164

Topical DNA oligonucleotide therapy reduces UV-induced mutations and prevents cancers in hairless mice

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UV irradiation causes DNA mutations in skin that may lead to skin cancer. We have shown that treatment of cells or skin with DNA oligonucleotides increases DNA repair capacity (DRC) through up-regulation of repair proteins. To investigate *in vivo* the effect of topical T-oligo treatment on DRC, UV-induced mutation frequency (MF) and carcinogenesis, we used hairless mice transgenic for the *lacZ*/pUR288 mutation-indicator gene. First, we evaluated the effect of thymidine dinucleotide, (pTT 100 μM) pretreatment (10 days) on the removal of cyclobutane pyrimidine dimers (CPDs) and MF after a single dose of UVB (30 mJ/cm^2). By 72 hours no CPDs remained in the skin of pTT-treated mice, whereas in diluent-treated animals 15±4% of CPDs remained ($p<0.05$). Moreover, compared to control, pTT treatment decreased the UV-induced MF by 31±5% ($p<0.03$). Mice then received 5 consecutive cycles (150 days) of one week of daily topical pTT (or vehicle alone) treatment followed by 3 weeks of daily UVB; 160 days after the initial UV irradiation mice were evaluated for tumor incidence, size and multiplicity, histology, as well as MF. pTT pretreatment significantly decreased the prevalence of malignancies between weeks 6 and 24 after initial UVB exposure. By 160 days 100% of control mice had tumors vs 25% of pTT-treated mice ($p<0.036$). There was a 91% decrease in the average number of tumors/mouse (3±1.3 vs. 0.25, $p<0.05$) in pTT vs diluent-treated mice. No tumors developed in sham irradiated mice. Compared to diluent, pTT treatment decreased by 28% MF ($p<0.08$) as assessed in irradiated non-tumor-bearing samples 160 days after initial UVB. We conclude that topical pTT treatment of mouse skin enhances DRC, decreases mutation frequency and carcinogenesis following UV irradiation. Our data suggest that topical DNA oligonucleotide therapy may prevent malignancies due to UV and other environmental carcinogens.

0165

Hyperphosphorylation of p70S6k and ribosomal protein S6 in tuberous sclerosis skin tumors is inhibited by rapamycin

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Patients with tuberous sclerosis complex (TSC) possess inactivating mutations in the *TSC1* or *TSC2* genes that predispose to developing tumors. Recently it was shown that these tumor suppressor genes function to inhibit the mTOR signaling pathway, a mitogen-activated pathway that stimulates cell growth through phosphorylation and activation of p70S6k and its substrate ribosomal protein S6. The aims of this study were to determine whether the mTOR pathway is activated in TSC skin tumors, and whether hyperactivity can be inhibited by rapamycin, an inhibitor of mTOR being tested for treatment of TSC internal tumors. In immunohistochemistry studies, cells immunoreactive for phospho-p70S6k and phospho-S6 were observed scattered in the dermis of 1 angiofibroma and 2 periungual fibromas, but not in the dermis of normal appearing skin. In Western blot studies, total and phosphorylated p70S6k and S6 were measured in cells serum starved for 48 hours to remove serum stimulation of the mTOR pathway. Phospho-p70S6k and phospho-S6 were faint or undetectable in fibroblasts cultured from TSC normal appearing skin, and detectable as moderate to intense bands in stromal cells from 3 angiofibromas, 1 periungual fibroma, and 1 shagreen patch. The addition of rapamycin (200 nM) to TSC tumor stromal cells for 24 hours dramatically reduced or eliminated phosphorylation of p70S6k and S6 in all three types of TSC skin tumors. Immunocytochemistry showed that 20 nM rapamycin decreased or eliminated cytoplasmic staining for phospho-p70S6k or phospho-S6 in cells cultured from 2 angiofibromas and 1 periungual fibroma. These data indicate that TSC skin tumors show increased activation of the mTOR signaling pathway, and that rapamycin inhibits this growth stimulatory pathway. TSC skin tumors may be useful as readily accessible surrogates to demonstrate adequate drug levels in ongoing clinical trials of rapamycin for TSC internal tumors.

0166

BRAF exon 15 mutations in melanoma: presence in primary melanomas and evidence for constitutive ERK activation in the absence of mutation

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Mutations in the BRAF gene (predominantly T1796A yielding V599E) were recently reported in a high proportion of melanoma cell lines and metastases (Davies et al., Nature 417:949). However, that study presented no data on primary melanomas. In preliminary experiments, we have detected BRAF exon 15 mutations in 14 of 66 (21.2%) primary melanomas. We also found the BRAF T1796A mutation in 11 of 21 (52.4%) metastatic melanomas and in 13 of 21 melanoma cell lines (61.9%). Two of the 21 lines contained a GT1795-96AA (V599K) mutation. To ask whether BRAF mutations act through the MEK-ERK pathway to stimulate growth, we treated normal human melanocytes (NHM) and 12 melanoma lines with the MEK inhibitor U0126. Cells (5,000/cm2) were seeded in 96-well dishes and maintained for 4 d in 0 to 10 uM U0126. NHM displayed only limited growth inhibition (97.7 of control (%C) at 3 uM and 72.1%C at 10 uM). Growth inhibition was also limited in the 4 melanoma lines without exon 15 mutations (82.2+/-5.5%C at 3uM and 70.7+/-2.6%C at 10uM). BRAF mutant lines displayed marked variability in growth inhibition (15 to 119%C at 3 uM, and 25-101%C at 10 uM). Only 3 of the 8 mutant lines tested were inhibited by >50%. By immunoblotting, phospho-ERK remained elevated in all melanoma lines after prolonged (24-72 hr) elimination of serum from DMEM, whereas it was markedly reduced (by 60-95%) in NHM after prolonged removal of the growth factor supplement from M154. These results demonstrate the presence of BRAF mutations in primary as well as metastatic melanomas. They also demonstrate that melanoma cell lines, unlike normal melanocytes, maintain a high level of signaling through the ERK pathway in the absence of growth factors, irrespective of BRAF exon 15 mutation status. However, the fact only 3 of 8 lines carrying exon 15 mutations were growth-inhibited by U0126 indicates that inputs independent of or distal to MEK are also involved in the proliferation and/or survival of melanoma cells.

0167

Immunophenotyping of cutaneous B cell lymphomas by flow cytometry

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Immunophenotyping of cutaneous B cell lymphomas (CBCL) by immuno- histochemistry has significant shortcomings. The panel of antibodies available for paraffin embedded tissue is limited. Conversely, there is an extensive panel of markers for frozen tissue, but processing artifact frequently hampers the interpretation. Our goal was to evaluate the feasibility of flow cytometric immunophenotyping of skin biopsies. We evaluated 37 skin biopsies from 36 patients with suspicious lesions for CBCL. Clinicopathological correlation was available on all cases. The samples were evaluated by 3-4 color cytometry techniques using variable panels depending on the number of cells extracted from the biopsy. Adequate cell suspension samples were obtained in most cases with mechanical pressure from a single 4mm punch biopsy. There were 6 cases of reactive lymphoid hyperplasia (B-cell type), 24 primary CBCL, including 9 cases of follicular center cell, 5 marginal zone and 10 large B-cell lymphomas (LBCL). There were 7 secondary CBCL (3 small lymphocytic, 1 diffuse small cleaved, 1 lymphoplasmacytic and 2 large cell lymphomas). Light chain restriction was detected in 77.4% (24/31) of lymphomas. All 6 RLH cases were polyclonal. We were unable to detect clonality on 7 lymphomas including 5 LBCL. Nevertheless,clonality was detected on 7/12 LBCL. Further evaluation with additional markers (CD5, CD10, FMC7, CD23, bcl-2) was helpful in subtyping the lymphomas. With the exception of LBCL which can yield either negative surface immunoglobulins or inadequate sampling due to cell fragility, flow cytometry is superior to immunohistochemistry(formalin fixed or frozen tissue) for immunophenotyping primary and secondary CBCL.

0168

Hemidesmosome integrity is required for invasive human epidermal neoplasia

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The recent generation of Ras-driven invasive human epidermal neoplasia resembling squamous cell carcinoma (SCC) provides the opportunity to study the functional importance of proteins overexpressed in SCC. These include the hemidesmosome components BP180, a6b4 integrin and laminin 5. These proteins promote cellular adhesion, growth and survival, however, their importance in carcinogenesis is unknown. To study this, we used cells from genetically characterized patients with junctional epidermolysis bullosa (EB) due to mutations in the COL17A1, ITGb4 and LAMB3 genes. Oncogenic Ras was co-expressed with IkBa in primary human keratinocytes deficient in these proteins and injected subcutaneously into nude mice to assay tumor formation. While normal controls produced rapidly growing tumors in 100% of animals within 2 weeks, Ras-IkBa expressing cells lacking either BP180, laminin 5 or a6b4 integrin all failed to produce any tumors (n=5/group). In each case, retroviral re-expression of the previously absent protein restored tumorigenicity, confirming that failure of tumor formation was due to its absence. In contrast, TGM1 mutant cells from patients with lamellar ichthyosis formed tumors with the same kinetics as controls, indicating that impaired tumor formation is not a non-specific feature of genodermatoses keratinocytes. Consistent with these genetic studies, injection of blocking antibodies to laminin 5 and a6b4 integrin also inhibited tumor formation while control IgG entirely failed to abolish tumorigenesis. Of interest, primary human keratinocytes from keratin 5 mutant EB simplex patients also failed to form Ras-driven tumors, suggesting that normal cytoskeletal interactions with hemidesmosomes might also be required for tumorigenesis. These data indicate that proteins necessary for hemidesmosome function are required for Ras-driven human epidermal neoplasia and provide a new genetic approach to study the role of individual proteins expressed in human cancer.

0169

Printed small molecule arrays for discovery of small molecules that interact with a protein of interest

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Discovery of small molecules that inhibit specific target proteins is typically a costly process requiring abundant purified protein and a robust bioassay. ATR is a protein kinase involved in the replication checkpoint for which a potent specific inhibitor would be of great interest. We have taken a novel approach to discover inhibitors for ATR, a large (301 kDa) protein that is not readily purified and for which no simple bioassay exists. Approximately 7,000 compounds from a diversity-oriented synthetic chemical library were robotically arrayed onto activated glass microscope slides to form covalently captured compounds. Cellular extracts were prepared from 293T cells that over-expressed Flag (epitope)-tagged ATR and from mock transfected cells. After sequential incubation with a mouse anti-Flag antibody and a fluorescently-labeled anti-mouse antibody, slides were read in a fluorescence scanner. Six small molecules reproducibly yielded a signal with lysates from Flag-ATR transfected cells but never with mock-transfected lysates. The compounds were structurally very similar to each other suggesting that they might recognize a common target (presumably ATR). The six compounds were tested for functional activity in a cell-based premature chromatin condensation assay and four of the six demonstrated inhibition of the ATR pathway. The most potent of these is being studied with structure-function analysis and in vitro kinase assays. Printed small molecule arrays have thus far been used successfully with purified proteins. This is the first application of this new technology to a target protein in a cellular lysate. This efficient approach has the potential to allow discovery of small molecules relevant to many biological systems.

0170

ATR regulates chromosomal fragile sites by enforcing the replication checkpoint

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The replication checkpoint is required for the response to UV and other forms of DNA damage by ensuring that DNA replication is complete prior to mitosis. Here we describe studies that reveal greater detail of the role of the protein kinase ATR (ataxia telangiectasia and Rad3-related) in the replication checkpoint and genomic fidelity. We inhibited ATR function using small interfering RNA (siRNA; duplexed 21-nucleotide RNA oligos of identical sequences to ATR). While control RNAs had no effect, siRNA to ATR caused a several-fold inhibition of ATR protein expression and partial functional inhibition of ATR. Specifically, siRNA increased sensitivity to caffeine (an inhibitor of ATR), augmented premature chromatin condensation, and induced chromosomal gaps and breaks at classically defined 'fragile sites' in cells treated with aphidicolin (slows DNA replication). Because cancer cells often have insertions and translocations at these same fragile sites, this finding suggests that ATR function may be limiting in malignant cells. To study ATR's role in skin in vivo we generated transgenic mice using a keratin-14 promoter linked to dominant negative alleles of either ATR or its downstream target Chk-1. Five independent founders carrying one of these transgenes (3 of ATR; 2 of Chk-1) are viable and without apparent defects in skin or hair at 4 months of age. This suggests that partial inhibition of the replication checkpoint is well tolerated in the absence of DNA damage. These findings suggest that ATR plays a key role in maintaining genomic fidelity by preventing premature chromatin condensation, an event that occurs mostly at 'fragile sites' that replicate late in S phase, and that partial inhibition of ATR function would be expected to be more detrimental to malignant than benign cells.

0171

Alternative splicing of 25-hydroxyvitamin D3-1α-hydroxylase (Cyp27B1) in malignant melanoma

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In vitro studies have demonstrated that the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) suppresses proliferation and induces differentiation in various cell types, including melanoma cells. There are two hydroxylases involved in the formation of circulating 1,25(OH)2D3 Theses hydroxylases belong to a class of proteins known as cytochrome p450 mixed function monooxidases. 25-hydroxyvitamin D3-1α-hydroxylase catalyses the synthesis of 1,25(OH)2D3 from 25-hydroxyvitamin D3 in the kidneys. Previous reports have shown that 1,25(OH)2D3 is also synthesized in cells from various extra-renal tissues including keratinocytes and activated macrophages. Increasing evidence indicates that extra-renal 25-hydroxyvitamin D3-1α-hydroxylase may act in many tissues via the local production of 1,25(OH)2D3 as a major regulator of cell growth in an autocrine or paracrine fashion. In this study we show the expression of 1α-hydroxylase for 25-hydroxyvitamin D3 in primary cutaneous malignant melanoma and in various melanoma cell lines and report the expression of several alternatively spliced 1α-hydroxylases mRNAs including transcripts encoding truncated proteins. Additionally, the regulation of these newly detected splicevariants by various vitamin D analogs was analyzed. Alternative splicing is a frequent feature in the expression of many p450 genes and is considered as an important factor in regulating the enzyme level. This is the first report of alternatively spliced mRNAs of the 1α-hydroxylase gene in melanoma that influence the 1α-hydroxylase activity level and the local synthesis of vitamin D metabolites in these cells.

0172

The first detailed study of multiple forms of mtDNA damage in NMSC

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Mitochondrial DNA (mtDNA) damage, predominantly encompassing point mutations, has been reported in a variety of cancers. We present in human skin, the first detailed study of the distribution of multiple forms of mtDNA damage in non-melanoma skin cancer (NMSC) compared to histologically normal perilesional dermis and epidermis. It includes the first entire spectrum of deletions found between different types of skin tumours and perilesional skin (n=30). Using long extension PCR to amplify the entire mt genome, 20 different deletion species were observed. Quantification by real-time PCR assay showed more deletions occured in the BCC (mean %-deletions=25.1±13.2) compared to SCC (mean %-deletions=12.3±18.0) (p=0.036, 2 sample t test) patients. We also performed the first quantitative analysis of the common deletion as well as the first identification of specific tandem duplications in tumour and the normal perilesional skin. Furthermore, DNA sequencing of 4 mutation 'hotspot' regions of the mt genome in 30 samples identified several mutions, e.g. there was an unreported somatic heteroplasmic C to T transition at np438 in a 77-year-old SCC patient. Moreover, there were two heteroplasmic changes, a T to C transition at np16093 in the non-coding D-loop and an A to G transition at np215 in the coding region, in the tumour of an 88-year-old BCC patient which were absent in the perilesional dermis and barely detectable in the associated epidermis. 81 unreported and reported homoplasmic single base changes were also identified in the other NMSC patients. However these mutations were present in both tumour and perilesional skin, which suggests that the use of histologically normal perilesional skin from NMSC patients, may be limited. In summary, it is unclear whether mtDNA damage has direct causal link to skin cancer or it may reflect underlying nDNA instability.

0173

Cutaneous T cell lymphoma: allelic loss at 10q23.33 and Fas gene mutations

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Chromosomal abnormalities at 10q have been identified in mycosis fungoides (MF) and Sezary Syndrome (SS) using cytogenetic techniques. Fas (CD95/Apo-1) gene mutations have been identified in other lymphoproliferative disorders resulting in defective lymphocyte proliferation. This study fine maps the area of deletion on chromosome 10q using loss of heterozygosity (LOH) and analyses Fas, a candidate gene within the minimal region of deletion, using PCR/SSCP analysis. In patients with MF (45) and SS (15), DNA extracted from either skin biopsies or peripheral blood and matched unaffected tissues (controls) was analysed for LOH at each of 19 microsatellite markers in the region 10q22.3-10q26.13. Allelic loss was found in 26/60 samples (43%). 15/45 (33%) patients with MF had LOH (1/3 - stage Ia disease, 1/8 - Ib, 12/29 - IIb and 1/3 - IVa). LOH was found in 11/15 (73%) patients with SS. The pattern of allelic loss was similar in SS and MF. Two minimal regions of deletion (MRD) were found: the first at 10q23.33 (between microsatellite markers D10S583 and D10S571) and the second at 10q25.3-10q26.11 (between microsatellite markers D10S187 and D10S221). PCR/SSCP using 13 primer pairs spanning the 9 exons of Fas was performed on 40 DNA samples (20 MF and 20 SS). Band shifts were seen in 6/40 (15%) patients (2/20 MF, 4/20 SS). 4/6 band shifts were in exon 1 and 2/6 in exon 3. 3/6 samples demonstrating band shifts in Fas also had LOH at 10q23.3 suggesting biallelic inactivation of Fas. DNA sequence analysis is being performed to determine if the observed band shifts represent novel Fas gene mutations. This extensive allelotyping data defines 2 MRDs on chromosome 10q in CTCL which provide the basis for targeting further candidate gene analysis. Results suggest that the Fas gene is an important potential target on 10q for gene inactivation in CTCL.

0174

Description of a novel antigen expressed in activated dermal cells and at the tumor–stroma–border of dermal tumors *in situ*

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In previous investigations we isolated a novel cDNA that is induced in fibroblasts by supernatants from malignant melanoma cell lines in vitro. This cDNA, Mel4b3, is induced by TGFbβ and Interleukin-1 in fibroblasts, keratinocytes and endothelial cells in vitro. The mRNA was detected in situ in skin biopsies of malignant melanoma, sqamous cell carcinoma, but not in benign naevi or normal skin. The tumor associated expression was mostly found at the tumor–stroma border. Interestingly, often cells of the outer root sheath of the hair follicle expressed this mRNA also in healthy skin. Sequence comparison revealed high homology to KIAA1866, a model reference sequence that was predicted from NCBI contig NT-029991 by automated computational analysis. However, the predicted protein coding sequence of KIAA1866 was distal from the open reading frame found in our sequencing work for Mel4b3. We developed polyclonal antibodies against peptide sequences of Mel4b3 and screened the expression of the mRNA as well as of the detectable antigen in order to test whether this peptide co–localizes with the mRNA message in probes from normal skin and skin tumors, especially malignant melanoma. Using in situ hybridization and immunohistochemistry we could show that the predicted peptide obviously exists and the immunohistochemical data clearly co–localize with mRNA expression in the skin specimen investigated. From these studies we conclude that the corresponding antigen might be associated with cell activation found in tumor development and hair growth.

0175

Loss of PKC-alpha expression may enhance the tumourigenic potential of Gli1 in BCC

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Aberrant signalling of the SHh pathway is associated with the formation of several neoplasms including BCC; this is primarily due to mutation of the PTCH1 tumour suppressor gene. Although PTCH1 mutations have not been identified in all BCCs examined, Gli1 is consistently upregulated suggesting that other factors may be relevant to tumour formation. Recent evidence has shown that Gli1 induces expression of Wnt isoforms and PDGFR alpha thus showing that SHh signalling is linked to other major signalling pathways. However, apart from known components of the SHh pathway that regulate Gli1 (such as PKA), little is known about other aspects of cell signalling which may influence Gli1 activity. Therefore, we determined the expression profile of two PKC isoforms in BCC sections and also their effect on Gli1 activity. We observed that neither PKC Alpha nor PKC Delta are expressed in tumour islands. In BCC sections containing hair follicles PKC-A is strongly expressed in the outer root sheath (ORS) whereas PKC-D is predominant in the inner root sheath. As we have previously shown that Gli1 is expressed in the ORS, we employed a luciferase reporter assay to determine if PKC-A regulates Gli1 activity; we also determined if PKC-D regulates Gli1 activity. In mammalian 293T cells, co-expression of constitutively active (ca) PKC-A reduced Gli1 activity by over 60% whereas ca-PKC-D increased activity over 2-fold. Western analysis demonstrated that the increase in Gli1 activity induced by PKC-D was not due to an increase in Gli1 protein. Surprisingly, despite reducing Gli1 activity, PKC-A actually increased Gli1 protein indicating that Gli1 activity would be further reduced if the values were also normalized to protein levels. The origin of BCC is uncertain but immunostaining studies have implicated that it may arise from the ORS of the hair follicle. We propose that loss of PKC-A expression may be important to BCC formation; this may in part be due to the predicted increase in Gli1 activity.

0176

The vitamin D system in malignant melanoma

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We analyzed key components of the vitamin D system in malignant melanoma (MM) in situ using immunhistochemistry and in melanoma cell lines (BuHom, MelJuso, MelJu, MeWo, A375) in vitro using real-time PCR (LightCycler). Additionaly, effests of the vitamin D analogs on growth and on expressioin of key components of the vitamin D system was analysed in melanoma cell lines. We detected strong vitamin D receptor (VDR) immunoreactivity in MM. Comparing VDR staining with staining for Ki-67, or apoptotic cells (terminal UTP nucleotide end labelling assay), no correlation was found. In melanoma cell lines, the majority of tumor cells revealed VDR immunoreactivity in vitro. Incubation of MelJuso cells with 1,25(OH)2D3 resulted in a reduction of the number of Ki-67-positive tumor cells and a reduced incorporation of pyrimidine analogue BrdU into the DNA of proliferating cells, indicating responsiveness of these cell lines to the antiproliferative effects of 1,25(OH)2D3. Using real time PCR, we have quantified mRNA expression of vitamin D receptor (VDR) and of vitamin D-25-hydroxylase, 25-hydroxyvitamin D-1α-hydroxylase, 25-hydroxyvitamin D-24-hydroxylase. Expression levels were determined as ratio between target genes and the reference gene PBGD. Interestingly, all enzymes were deected in melanoma cell lines. Our findings indicate that (i) VDR protein as well as mRNA for VDR, 25-OHase, 1α-OHase and 24-OHase are strongly expressed in MM. (ii) VDR expression in MM is not exclusively regulated by the proliferative activity of these tumor cells, but additional, unknown mechanisms (iii) alterations in VDR expression and in the synthesis and metabolism of vitamin D metabolites may involved in the growth regultion of MM (iv) MM may be considered as potential target for therapy with new vitamin D analogs that exert little calcemic side effects or by pharmacological modulation of 1,25(OH)2D3 sythesis/metabolim in these tumor cells

0177

Immunolocalization of low affinity PGE_2 receptors, EP_1 & EP_2, in human epidermis and non-melanoma skin cancer

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In this study, we examine the immunolocalization of the low-affinity PGE_2 receptors, EP_1 and EP_2, in normal human epidermis and human squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). Cyclooxygenase-2 (COX-2) expression is markedly elevated in non-melanoma skin cancers. Overexpression of COX-2 as well as treatment with COX-inhibitors results in decreased invasive behavior in a variety of tumor cell lines. We have previously reported that loss of the EP_2 subtype of PGE_2 receptor results in increased invasiveness in spontaneously immortalized HaCaT cells [Am. J. Pathol., 161:2079-2086, 2002]. We have therefore hypothesized that PGE_2-mediated down-regulation of low-affinity PGE_2 receptors may be involved in acquisition of the invasive phenotype. Using two monoclonal antibodies developed against the human EP_1 and EP_2 receptors, we performed immunohistochemical staining of normal human epidermis and non-melanoma skin cancers. In normal epidermis, EP_1 and EP_2 receptors both demonstrated perinuclear and cytoplasmic or cell membrane localization. Both demonstrated increased staining in the stratum granularis, with scattered sparing of the basal compartment. Eccrine glands, smooth muscle, hair follicles, and sebaceous glands all stained positive for both EP_1 and EP_2 receptors. EP_2 receptor staining was variably decreased to absent in 4/5 SCC, increased in 1/5 SCC and absent in 3/3 BCC. EP_1 immunostaining was decreased to absent in 6/6 SCC and absent in 1/1 BCC. In general, areas of tumors which stained positive were more superficially located or in more differentiated areas. Areas of deeper penentration and highly cellular areas generally demonstrated decreased to absent immunostaining. These results examining a small series of non-melanoma skin cancer strongly support our hypothesis that loss of low-affinity PGE_2 receptor expression is associated with acquisition of the invasive phenotype.

0178

Specific T helper cells selectively inhibit tumor angiogenesis through TNF-receptor1-dependent signaling

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RIP1-Tag2 transgenic mice are an established model for multistage tumorigenesis and angiogenesis. In these mice the large T antigen (Tag) of the Simian Virus 40 is only expressed in insulin-producing β cells of the pancreas, leading to the successive development of islet cell hyperplasia, adenomas, and finally invasive, highly vascularized carcinomas. As we recently found that adoptive transfer of Tag-specific Th1 cells is highly efficient in inhibiting tumor growth, we investigated the underlying mechanisms, including the role of Tumor necrosis factor (TNF) signalling. At 6 weeks, RIP1-Tag2+TNF-receptor1 (TNFR1)-/- mice were treated with Tag specific Th1 cells weekly. Th1 cells were generated by stimulating Tag-specific, T cell receptor-transgenic CD4+ T cells from C3H mice with Tag and antigen presenting cells in the presence of CpG 1668 oligonucleotide. We followed blood glucose levels and performed histology. To evaluate, whether TNFR1 signaling interferes with the anti-angiogenetic effect of Th1 cells, we used a glycosylated RGD-peptide with selective binding to integrin αvβ3, a marker for endothelial cell activation and angiogenesis. Adoptive transfer of Th1 cells doubled life span of RIP1-Tag2 positive TNFR1+/+ mice. In sharp contrast, Tag-specific Th1-cells did not prolong life of RIP1-Tag2+TNFR1-/- mice. Importantly, at 12 weeks TNFR1-/- mice had large, vascularized islet carcinomas, whether they were treated with Th1-cells or not. Quantifying angiogenesis in vivo with radio-labeled integrin αvβ3 proved that Tag-specific Th1 cells prevented activation of integrin αvβ3 in tumors of normal RIP1-Tag2+ mice, while it failed to inhibit αvβ3 in RIP1-Tag2+TNFR1-/- mice. As we recently found that tumor-specific Th1 cells efficiently prevent tumor growth through mechanisms independent of tumor cell killing, the data provided here show that Th1 cells control tumor growth and angiogenesis through TNFR1-mediated signaling.

0179

Regulated expression of a dominant/negative AP-1 (A-FOS) targeted with the Keratin 5 promoter alters epidermal inflammation, tumorigenesis, and causes blindness

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To address the specific role of the AP-1 family in stages of skin tumorigenesis, transgenic mice were generated with a dominant negative Fos protein (A-FOS) under the control of the tetracycline operator region (tet-O-A-FOS). These mice were crossed with Keratin 5-Tet-transactivator mice, such that A-FOS is targeted to basal skin keratinocytes and hair follicle outer root sheath in the absence of doxycycline (Dox) in bi-genic mice. Tumors were induced in bi-genic and control (single transgenic and bi-genic on Dox) mice by a single application of DMBA followed by repeated application of PMA. Unexpectedly, bi-genic mice without Dox developed twice as many benign lesions as bi-genic mice on Dox or single transgenic mice. However, tumors on bi-genic mice off of Dox were small sebaceous adenomas, whereas tumors in all control groups were squamous papillomas or carcinomas. When papilloma bearing A-FOS mice on dox were placed on normal feed, the rate of malignant conversion was reduced and tumors had mixed sebaceous and squamous components. PMA treatment alone resulted in an inflammatory infiltrate, basal cell apoptosis and altered gene expression profiles on chip based microarrays in bi-genic mice off dox compared to controls. Aged bi-genic mice off Dox develop eye reddening and alopecia, a syndrome compatible with vitamin A deficiency that improved by implantation of time-release retinoic acid pellets, though was not completely reversible. Aged bi-genic mice also were blind and lacked rods and cones, possibly secondary to K5-A-FOS expression in the retinal pigment epithelium. Consistent with an impact on retinoid pathways, changes in the expression of retinoid metabolizing enzymes and binding proteins were detected on cDNA microarrays of bi-genic skin. Together, these studies provide insight into the role of AP-1 in skin tumorigenesis and the interplay between retinoid and AP-1 pathways.

0180

A model system for studying $p16^{INK4a}$ expression

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The $p16^{INK4a}$ gene product is a major mediator of replicative senescence and functions as a tumor suppressor. $p16^{INK4a}$ expression is often lost in melanoma through deletion, mutation or hypermethylation of its promoter. Furthermore, methylation of the $p16^{INK4a}$ promoter is known to decrease as cells age. We have shown that oligonucleotides homologous to the telomere 3' overhang (T-oligo) activate multiple, anti-proliferative and cancer-prevention cellular response and that a one week exposure of human fibroblasts (fb) to T-oligo induces a senescent phenotype, mimicking replicative senescence induced by serial passage in vitro. In order to develop a model to study $p16^{INK4a}$ gene regulation during senescence and carcinogenesis, normal human fb were treated with the T-oligo pGTTAGGGTTAG, complementary control oligo or diluent alone for up to seven days and then collected for analysis. Western blot showed that $p16^{INK4a}$ protein was selectively first induced 5 days after T-oligo treatment and remained elevated through 7 days. $p16^{INK4a}$ was also induced 5 days after transfection of fb with a dominant-negative telomere repeat binding factor, $TRF2^{DN}$, known to cause telomere disruption. Western analysis further showed that T-oligo induces the Ets-1 transcription factor, a known regulator of the $p16^{INK4a}$ promoter, but interestingly within 24 hours, much earlier than the observed $p16^{INK4a}$ protein induction at day 5. Furthermore, a transfection of fb with a $p16^{INK4a}$ promoter luciferase construct revealed a 4 to 5 fold increase in promoter activity within 48 hours of T-oligo treatment. These data suggest that exposure of the telomere 3' overhang induces transcription of $p16^{INK4a}$ protein expression via Ets-1, but that $p16^{INK4a}$ induction lags behind promoter activation by 48-72 hours, consistent with a requirement for promoter demethylation. T-oligos appear to mimic a physiologic signal for $p16^{INK4a}$ induction and may provide a valuable model for exploring its altered regulation in melanoma.

0181

A novel approach to melanoma therapy using telomere overhang-induced DNA damage signals

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Melanoma, the most fatal form of skin cancer, is highly resistant to chemotherapy. We have shown that treatment of the aggressive MM-AN human melanoma cell line with an 11-base oligonucleotide homologous to the telomere 3' overhang sequence (T-oligo) induces cell cycle arrest, apoptosis, and differentiation of these cells. This apoptotic response is mediated at least in part by the p73 tumor suppressor. To further elucidate the mechanism of T-oligo effects, we examined expression of livin/ML-IAP an anti-apoptotic protein over-expressed in melanoma. By western blot, T-oligo but not a control complementary oligo or diluent alone, dramatically down-regulated livin. Furthermore, T-oligo enhanced the apoptotic response of MM-AN cells to cisplatin. To determine the effect of T-oligo on MM-AN tumorigenicity and metastasis in SCID mice, 3 groups of 5 mice each were injected with cells pretreated for 48 hrs with T-oligo, complementary or diluent. After 40 days, the average number of metastatic tumors in the control groups were 15.8 and 9.6, compared to 0.8 in the T-oligo pretreated group ($p<0.05$). Tumor volume was reduced 85-95% and 4 of the 5 mice receiving the T-oligo treated cells were free of detectable metastases. In order to determine whether T-oligo can affect the growth of pre-existing tumors, MM-AN cells were injected subcutaneously into the flank of SCID mice. Sites were injected twice daily with 60 nmoles of T-oligo or controls. At autopsy (day 22), there was no significant difference in tumor size between diluent and control oligo-treated mice, but T-oligo treatment reduced tumor volume by 84-88% ($p<0.05$), to clinically undetectable size in 3 of 6 mice. In tissue sections T-oligo treated tumors showed apoptosis (TUNEL staining) and greatly enhanced expression of the differentiation associated proteins gp100 and TRP-1. We propose that T-oligos mimic a physiologic DNA damage signal that is frequently masked in malignant cells and induces innate anti-cancer responses.

0182

DNA damage and senescence responses are induced by degradation of telomere-specific DNA

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During aging, telomeres gradually shorten and when critically short, cells die by apoptosis or enter a state of growth arrest known as senescence. Conversely, malignant conversion of cells occurs only when this safeguard is bypassed, usually through expression of telomerase. Senescence is associated with short telomeres, but the mechanism by which telomere shortening renders cells nonproliferative is unclear. Normally, telomeres form a loop structure with the 3' single-stranded overhang (tandem repeats of TTAGGG) protected within the duplex DNA. Upon experimental telomere loop disruption, this overhang is exposed, removed and the cells undergo DNA damage responses such as p53 induction, apoptosis or development of a senescent phenotype. We and others have shown that mammalian cells respond to DNA oligonucleotides homologous to the overhang (T-oligos) by induction of DNA damage responses, apoptosis or senescence, suggesting that exposure of the TTAGGG sequence is the salient event in loop disruption. We now report that T-oligos containing nuclease-resistant phosphorothioate (ps) backbone linkages are inactive and do not induce a cell cycle arrest or apoptosis in Jurkat cells as do physiologic nuclease-sensitive phosphodiester (pde)-linked T-oligos. Confocal microscopy revealed identical cellular uptake and nuclear localization of fluorescently-labeled ps- and pde-T-oligos, but ps-T-oligos did not induce phosphorylation of p53-serine 15 or the S-phase checkpoint protein p95/Nbs1, as do pde-T-oligos. In order to further study the basis of activity, we constructed 11-base T-oligos with ps linkages between the first 3 bases (2 linkages) either only on the 3' terminus, the 5' terminus, or both termini. T-oligos blocked at both termini were inactive but opening one terminus to nuclease digestion restored their activity with a preference for an open 3' end, as would be the case after overhang exposure in vivo. These data provide the first experimental evidence that the telomere-mediated DNA damage and senescence responses require digestion of the exposed 3' overhang.

0183

Smad3 knockout mice exhibited resistance to chemically-induced skin carcinogenesis which correlated with the lack of TPA-mediated AP-1 activation

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Smad3, a signaling mediator of transforming growth factor β, has been suggested to be a tumor suppressor. To evaluate the role of Smad3 in skin carcinogenesis, we applied a skin chemical carcinogenesis protocol to Smad3 knockout mice (Smad3-/- and Smad3+/-) and their wildtype littermates (Smad3+/+, wt). Dimethylbenz[a]anthracene (DMBA) was applied to 3-day-old mouse skin, followed by applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) twice a week for 30 weeks. Thirty weeks after DMBA initiation, 87% wt mice developed skin tumors, most of which were papillomas with a few squamous cell carcinomas (SCCs). Surprisingly, only 6.7% Smad3-/- mice developed tumors which were all papillomas. Smad3+/- mice also exhibited reduced tumorigenesis (47%) and fewer SCCs in comparison with wt mice. Further analysis revealed that Smad3 knockout mice were resistant to TPA-induced epidermal hyperproliferation and tumor promotion. To further analyze the molecular mechanisms of the TPA resistance in Smad3 knockout skin, we examined the major AP-1 family members. TPA application significantly induced levels of c-Jun, JunD and c-Fos in wt skin. In contrast, these proteins were not elevated in Smad3-/- skin, and the levels of AP-1 proteins in Smad3+/- skin were only elevated up to 50% of those in wildtype skin. TPA application also induced phosphorylation and nuclear translocation of AP-1 family members in wt skin. However, both phosphorylation and nuclear translocation of AP-1 proteins were not detectable in TPA-treated Smad3-/- and Smad3+/- skin. Taken together, our study suggests that Smad3 may be required for TPA-mediated AP-1 activation. Thus, the lack of AP-1 activation in the epidermis may partially confer a resistance to chemically induced skin carcinogenesis to Smad3 knockout mice.

0184

Clusterin inhibits keratinocyte growth *in vitro* and reduces skin tumorigenesis *in vivo*

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Clusterin (ApoJ, SP-40,40) is a ubiquitous secretory glycoprotein that is known to suppress certain forms of apoptosis. It is produced by keratinocytes in vitro and expressed by suprabasal differentiating keratinocytes in vivo, and can be induced by TPA. Here we show that recombinant clusterin inhibits the growth of primary human keratinocytes in a dose dependent manner. The growth inhibitory effect of clusterin is also observed in other primary epithelial and endothelial cells but not fibroblasts. In accordance with this, anti-sense transfected clusterin A431 cells, that lack endogenous clusterin, show a significant growth advantage over mock-transfected and wild-type A431 cells in vitro. Microarray analysis of keratinocyte gene expression following clusterin exposure suggests that clusterin modulates the expression of several key genes implicated in the control of keratinocyte growth and differentiation. The in vivo role of clusterin was investigated in a chemical (DMBA/TPA) skin tumorigenesis model using wild type (WT) and clusterin-knock out (KO) mice. In this model, the mean number of papillomas per mouse was significantly increased in KO compared to WT mice. In conclusion, our results show that clusterin inhibits keratinocyte proliferation in vitro and early stages of skin tumorigenesis in vivo.

0185

Interrupting activated Notch signaling triggers apoptosis in melanoma cells

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Malignant melanoma (MM) cells are difficult to kill. This study explored Notch signaling in MM. Immunostaining melanocytes (MC) in normal skin (n=10) revealed no Notch receptor expression. Notch-2 immunostaining revealed strong expression in 7/22 (32%) benign nevi, 10/19 (52%) cutaneous MM, 56/76 (74%) cases of metastases. Notch-4 staining was strong in 14% of nevi, 95% of cutaneous MM, and 70% of metastases. Staining to detect Notch-1 or Notch-3 was not consistently positive in benign or MM lesions. Western blotting of MM lesions (n=4) confirmed overexpression of the activated (75 kd) and precursor (125 kd) forms of Notch-4 in all cases; whereas only precursor forms (110 kd) of Notch-2 were detectable. Compared to MCs, activated Notch-4 was overexpressed in MM lines. Interrupting Notch activation was accomplished using a gamma secretase inhibitor (GSI). Exposure to GSI of early passage MM lines (n=5), and established MM lines (n=5); produced concentration and time dependent induction of apoptosis amongst MM cells with 2 lines relatively resistant (< 5% apoptosis, 18 hrs; 10 μM), but all other lines were sensitive (20-40% apoptosis). GSI reduced activated Notch-4 levels within 2-4 hrs; accompanied by accumulation of transmembrane form. Serial biochemical analysis before, and beginning 6 hr after GSI exposure, revealed apoptotic machinery included activation of caspases 3,7,8,9, BID with release of SMAC and cytochrome C from mitochondria. Caspase inhibitors blocked apoptosis. GSI triggered apoptosis was characterized by initial increase (1 hr) of phosphorylated p38 MAPK, followed 2 hr later by accumulation of p53 containing key phosphorylated residues. These results demonstrate Notch-4 as the predominately active receptor in MM. Interruption of Notch signaling using GSI triggers apoptosis in MM. We conclude that Notch signaling plays an important survival role in MM, and represents an attractive novel therapeutic target.

0186

Gene expression patterns for diagnostics, prognostics and biology of CTCL

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We have used cDNA arrays of peripheral blood mononuclear cells to identify gene expression patterns that are characteristic of patients with leukemic forms of cutaneous T-cell lymphoma, primarily Sezary syndrome. Highly overexpressed genes include Th2 specific transcription factors Gata-3 and Jun B both required for Th2 differentiation and IL-4 expression. Other highly overexpressed genes include integrin 1, expressed on skin homing T-cells, proteoglycan 2, a cell surface protein that binds L-Selectin and regulates chemokine function, the RhoB oncogene, the apoptosis inducing cytokine TRAIL and the Map Kinase inhibitor Dual-specificity Phosphatase 1. Highly down regulated genes include CD26 and Stat-4 as well as the Interleukin 1 receptors. Message for the actin-bundling protein plastin-T, a gene that is not normally expressed in lymphoid tissue, was detected in 35 of the 45 patients examined. Aberrant expression of the gene in CTCL T-cells may provide a unique new marker for diagnosis for a high percentage of patients. Expression of a panel of 4 of the significant genes, monitored by quantitative Real-time PCR identifies 100% of patients regardless of tumor burden suggesting a simple diagnostic test that monitors the expression levels of a small number of genes may be developed. Our analyses suggest that many similar panels may allow diagnosis with other sets of genes. We have extended these studies to the identification of genes whose patterns of expression were able to identify patients with poor prognosis. Although these patients are more difficult to recognize, we have identified 40 genes whose patterns of expression detect a class of patients within the 45 whom we analyzed, who will succumb within six months of sampling, regardless of their tumor burden. Since the number of genes queried in pilot studies was small, more informative genes for prognosis and responsiveness to therapy will be identified in studies with 15,000 genes in a larger number of patients.

0187

NRAS and BRAF mutations in primary human melanomas

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NRAS genes encoding activating mutations have been found in 4 to 40% of primary melanomas, and activating mutations encoded in the BRAF gene, downstream of RAS in the RAS-RAF-MEK-ERK-MAP pathway, were found in 59% of melanoma cell lines and 6 of 9 primary melanomas. We plan to extend this information by carrying out a population-based study of NRAS and BRAF mutations including possible etiologies and have analyzed a pilot series of 18 archived primary human cutaneous melanomas from the UNC Hospitals Pathology Laboratories. Mutational status of tumor cells from paraffin-embedded tissue sections was determined using SSCP (single strand conformational polymorphism) analysis, conducted at two different temperatures, followed by sequencing of samples showing band shifts on SSCP to identify affected codons and exact base changes. These melanomas, with Breslow depths ranging from 0.73 to 10.5 mm, were screened for mutations in and around NRAS codons 12,13,18 and 61 and within BRAF exon 15. BRAF exon 15, which encodes a kinase activation segment, was selected for this initial study because 4 of 5 BRAF mutations previously reported in primary melanoma were found in this exon. Of the 18 melanomas analyzed in this study, 6 were positive for NRAS and 4 were positive for exon 15 BRAF mutations. The NRAS and exon 15 BRAF mutations were found to be exclusive of each other in these melanomas, consistent with the possibility that exon 15 BRAF mutation may abrogate the need for NRAS activation in melanoma. All 6 NRAS mutations were at sites of putative pyrimidine dimers, implying a possible link between NRAS activation and ultraviolet exposure in melanoma. For all 4 BRAF positive tumors, surrounding areas of normal skin were negative for the BRAF mutation. Because the BRAF alterations are specific for the tumor, these findings are consistent with BRAF exon 15 mutations being somatically acquired mutations rather than germline polymorphisms.

0188

Detection of mitochondrial DNA mutations in non-melanoma skin cancer: possible genetic selection in tumorigenesis

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Mitochondrial DNA (mtDNA) mutations have been found in colon carcinoma and a variety of other human malignancies. Surprisingly, these mutations were often present in all of the mtDNA copies (homoplasmic) within the tumor cells. Proposed theories to explain the role of these mtDNA changes in tumorigenesis have included that they impart a possible selective growth advantage or that they arise by random stochastic segregation. We explored the role of mtDNA changes in non-melanoma skin cancer (NMSC). Paired tissue specimens of NMSC tumors and adjacent tumor-free margin skin were obtained from excisions in our clinic. DNA was isolated from the specimens for screening of the entire mitochondrial genome by multiplexed denaturing high-performance liquid chromatography (DHPLC,) a column-based method for detection of DNA sequence variation. PCR-amplified segments of the mtDNA from the tumors were annealed with the PCR products amplified from the margin skin, allowing for the formation of heteroduplexes in regions of DNA mismatch. Regions of the mtDNA that formed heteroduplexes were detected by DHPLC and sequenced to determine the specific nature of the mtDNA base changes. A variety of polymorphisms as well as homoplasmic point mutations in the mtDNA have been identified including protein synthesis mutations affecting the mtDNA-encoded rRNA subunits and mutations affecting specific subunits of NADH-ubiquinone oxidoreductase (complex I) and cytochrome c oxidase (complex IV). Additionally, long extension PCR detected an abundance of mtDNA deletions from the margin skin that were generally absent from the tumor specimens. We propose that the relative lack of mtDNA deletions within NMSC tumor specimens and the finding of a variety of mtDNA point mutation in NMSC tumors argues against the random segregation theory of mtDNA mutation homplasmy and suggests a role for genetic selection of specific mtDNA haplotypes in NMSC development.

0189

Immunization with the tumor antigen hedgehog-interacting-protein reduces and anti-rejection drug administration increases BCC formation in ptc1 +/- mice treated with ionizing radiation

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Basal Cell Carcinomas (BCCs) in humans or in ptc1 +/- mice all overexpress hedgehog target genes such as Gli1, Hedgehog interacting protein (Hip), platelet-derived growth factor alpha, and Ptc1 itself. Our survey of normal adult mouse tissues indicates that their level of Hip mRNA expression is much lower than is that in BCCs. Accordingly we have selected this protein for immunization to try to prevent the development of BCCs in ptc1 +/- mice treated with ionizing radiation. Recombinant Hip protein fragments were injected subcutaneously in combination with CpG adjuvant every two weeks for 5 months. Immunization in ptc1+/- mice was well tolerated and induced peptide-specific B and T-cell responses detectable by ELISA, Western Blot, delayed type hypersensitivity, and ELISPOT assays. The number of BCCs in mice immunized over the course of 5 months with 2 different HIP-derived peptides, PT27 and PT209, was reduced by 45% ($p<0.001$) and 36% ($p<0.01$), respectively. In contrast, the number of tumors was increased 2.1 fold ($p<0.0001$) in ptc1 +/- mice treated for 4 months with a combination of the anti-rejection drugs Cyclosporin A and Prednisolone at doses sufficient to cause immunosuppression. We conclude that immunization with proteins specifically upregulated by hedgehog signalling may be a novel preventive option for patients such as those with the basal cell nevus syndrome who have a high likelihood of developing large numbers of BCCs. In addition, these mice seem to provide a good model for anti-rejection drug enhancement of non-melanoma skin cancers in humans who have received organ transplants. More generally, these data suggest that the host s immune system may help control the development of BCCs and that this protective activity may be enhanced by immunization with tumor antigens.

0190

PLZF regulates and suppresses melanoma proliferation and tumor growth

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Melanoma is a malignant skin tumor with a poor prognosis. In melanoma cells, mutations and the abnormal expression of genes related to the cell cycle regulating pathway, such as p16INK4A/p15INK4, CDK4/6, and cyclinD, have been reported. These abnormal genes are thought to be involved in the pathogenesis of melanoma. We hypothesized that other cell cycle regulatory genes are also involved in melanoma. Therefore, we examined 13 human melanoma cell lines and found that PLZF (promyelocytic leukemia zinc finger) was absent in all 13 cell lines, whereas it was expressed in normal human melanocytes. PLZF was first reported in 1995 as an abnormal fusion gene in acute premyeloid leukemia, in which cyclinA transcription was accelerated (Chen et al., 1995). Although the function of PLZF fusion proteins has been well studied, the function of normal PLZF is not clear. Recently it has been suggested that PLZF works as a transcriptional repressor. To elucidate the biological meaning of the absence of PLZF in human melanoma, we generated four PLZF-transduced melanoma cells (375p, 397p, 526p, and 624p). Surprisingly, PLZF-transduction suppressed the proliferation of 375p, 397p, 526p, and 624p to 52, 45, 79, and 48% compared to vector-transduced melanoma cells, respectively. In PLZF-transduced melanoma cells, cyclinA and cyclinD mRNA expression was suppressed. Transplantation of both PLZF- and vector-transduced melanoma cell lines to SCID mice revealed that PLZF-transduced melanoma cells reduced tumor volume compared to vector-transduced melanoma cells (375p, 397p, and 526p to 8, 11, and 39% on day 30, and 17, 46, and 44% on day 60, respectively). Combined, PLZF regulates melanoma growth in vitro and in vivo. These data suggest that the absence of PLZF expression is related to unregulated melanoma growth, and that PLZF will be a target of gene therapy in melanoma.

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The N-terminal domain of IL-1a associates with chromatin and enhances tumor conversion in two-stage carcinogenesis

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IL-1a is stored in abundance in keratinocytes as an active 31 kDa precursor. Cleavage produces the proinflammatory 17 kDa C-terminal domain and a 16 kDa N-terminal pro-domain of unknown function. The pro-domain contains a nuclear localization signal and transforms fibroblastic cells in vitro. To examine its function in cutaneous epithelial cells, we expressed a proIL-1a-gfp fusion protein in cultured mouse and human keratinocytes and observed nuclear localization and association with chromatin. To test pro-domain function in vivo, transgenic mice were created that constitutively express the pro-domain in skin using the K14 promoter. In two K14/pro founder lines (ProA and ProB), skin was histologically similar to control littermates suggesting that transgene expression did not disrupt normal skin development or induce inflammation. Following application of the two-stage carcinogenesis protocol, the early kinetics of papilloma formation and tumor incidence for both Pro lines were similar to their littermate controls. However, the peak papilloma burden for the ProA mice preceded that of their FVB littermates by 9 weeks. A subsequent precipitous decline in benign tumor burden of the ProA mice was accompanied by a decrease in average carcinoma latency (6 weeks; $p=0.02$), a 2.5 fold higher conversion frequency at 30 weeks post-initiation, and an increase in tumor size ($p=0.03$), suggesting that pro-IL-1a acted to enhance malignant tumor formation. For the lower-expressing ProB line, a significantly higher average papilloma burden occurred early in promotion (week 16; $p<0.05$) but the time of the peak papilloma burden, maximum number of papillomas, and carcinoma formation were not significantly different from controls. These results suggest that pro-IL-1a plays a role in the conversion of benign to malignant lesions, potentially cooperating with mutated H-ras to promote the outgrowth of initiated cells. A thorough understanding of the determining factors and pathways regulating the contribution of pro-IL-1a to tumor conversion may provide a target for cutaneous tumor intervention.

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Polyphenols extracted from green tea reduce UV-induced apoptosis of keratinocytes

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Green tea chemoprevention has been a focus of research for several years, as a polyphenolic fraction isolated from green tea (EGCG) has been shown to inhibit the development of ultraviolet radiation (UV)-induced skin cancers and to mitigate UV-induced erythema, thymine dimers, Langerhans cell depletion and immune suppression. Because the mechanism for these protective effects may include modulation of UV-induced apoptosis, experiments were designed to investigate this possibility. The human epidermoid carcinoma cell line KB and normal human keratinocytes were exposed to EGCG (0.05-0.5%) either 5 hours before and immediately after, or only immediately after UVB irradiation (250 J/m^2). UVB-induced apoptosis was reduced in UVB-exposed cells treated with EGCG under both conditions as determined by a DNA fragmentation ELISA. These data were supported by increased caspase-3 activation, a protease involved in UVB-induced apoptosis. These observations would exclude the possibility that the protective effect of EGCG is due to absorbance of UVB wavelengths. It has also been observed that the immunostimulatory cytokine interleukin-12 (IL-12) reduces UVB-mediated apoptosis and sunburn cell formation. This effect appears to be due to a reduction of UVB-induced DNA damage, which is mediated via induction of DNA repair by IL-12. We therefore examined the ability of EGCG to induce IL-12. For that purpose, blood monocytes were obtained from human volunteers, exposed to EGCG and 24 hours later the amount of IL-12 released was measured by using an ultrasensitive IL-12 ELISA. EGCG significantly induced the secretion of IL-12 by monocytes. Based on these findings we speculate that EGCG might reduce UVB-induced apoptosis via induction of IL-12 and subsequent upregulation of DNA repair. It remains to be determined whether these effects are related to green tea inhibition of UV-induced skin cancer.

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DNA mismatch repair enzyme hMSH2 in malignant melanoma

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Human hMSH2 is a well characterized component of the DNA mismatch repair system that has been implicated in cellular mutagenesis, tumorigenesis and chemotherapeutic resistance of malignant melanoma. hMSH2 is directly involved in microsatellite instability in hereditary nonpolyposis colorectal cancer (HNPCC). Microsatellite instability has also been shown in malignant human skin tumors of epithelial and melanocytic origin. Like hMSH2, the p53 tumor suppressor protein plays an important role in maintaining the fidelity of the cellular genome. P53 mutations are frequent in a variety of cancers. We have investigated hMSH2 and p53 expression in melanocytic tumors. We found strong nuclear immunoreactivity for hMSH2 and p53 that was elevated in malignant melanoma and melanoma metastases as compared to acquired nevi. Using the reverse transcription-polymerase chain reaction, we detected strong hMSH2 mRNA expression in each of 8 melanoma cell lines analysed (highest amounts in SK-MEL-25 cells, lowest amounts in MML-I cells). A relevant stress that activates p53 function is UV-light. We showed that upon UV-irradiation p53 activates transcription of the human mismatch repair gene hMSH2. Additionally, we identified a response element for p53 in the hMSH2 promoter and could show that hMSH2 up-regulation critically depends on functional interaction with c-Jun. In conclusion, our findings indicate that hMSH2 is involved in p53 mediated pathways in response to stress, including UV-radiation in malignant melanoma. These mechanisms may be of importance for tumorigenesis and chemotherapeutic resistance of malignant melanoma.

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BRAF mutations in primary melanoma vary with site and age, and are not associated with outcome

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The serine/threonine kinase BRAF has been reported to be frequently activated by somatic mutation in melanoma. To date no information is available on the distribution of BRAF mutations among different types of melanoma and their association with outcome. We sequenced the kinase domain in exon 15 of BRAF in DNA extracted from paraffin blocks of 116 invasive primary melanomas of four categories: 44 from skin without evidence of severe solar elastosis as an indication of chronic sun damage (CSD), 10 from skin with CSD, 41 from glabrous (non hair-bearing acral) skin, and 21 from mucosa. Clinical follow-up information was available from 89 patients with an average follow-up time of 37 months. The proportion of tumors with mutations varied significantly ($p<0.01$) between groups: 52% (23/44) on sun-exposed skin without CSD, 17% (7/41) on glabrous skin, 10% (1/10) on skin with CSD, and 9.5% (2/21) on mucosal sites. Independent of tumor class we found that patients with BRAF mutations were significantly younger ($p<0.026$). The age dependence was striking and indicated that the increased incidence of melanoma with age was due primarily to the increase of tumors without BRAF mutations. There was no association between mutation status and tumor thickness, metastasis, or death from disease. Array-CGH of 68 of the cases found that all cases with a mutation that had increased copy number of the BRAF region had selectively increased the mutated allele. This indicates that BRAF mutations precede the copy number increase of chromosome 7 and are one of potentially several genes driving this frequent aberration. This observation together with the lack of association with tumor thickness and outcome suggests that BRAF mutations occur relatively early in melanoma. In summary, our data indicate different genetic routes to melanoma that depend on anatomic site and sun-exposure patterns and suggest that melanomas without BRAF mutations may take longer to develop.

0195

Opposite roles of protein kinase C isoforms in regulating human HaCaT keratinocyte proliferation, differentiation, and tumor genesis

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The protein kinase C (PKC) system plays crucial roles in the regulation of proliferation, differentiation, and tumor formation of several cell types. In this study we examined the specific roles of PKC isoforms in the human keratinocyte cell line HaCaT, which possess several PKC isozymes (the conventional cPKCα, β; the novel nPKCδ, ε, θ, η; and the atypical aPKCζ). Expressions of differentiation markers were investigated by Western blotting, whereas the proliferation of the cells was assessed by a bromo-deoxy-uridine cell proliferation ELISA kit. Tumor genesis was assessed by intradermally injecting HaCaT cells overexpressing certain PKC isozymes to immunodeficient mice. Inhibition of cPKC and nPKC isoforms by GF109203X resulted in suppression of late-terminal differentiation but had no effect on proliferation. However, Go6976 (inhibitor of the cPKC isoforms) and Rottlerin (the inhibitor of nPKCδ), besides both inhibiting differentiation similarly to the effect of GF109203X, respectively suppressed or stimulated cell growth. Consistent with these findings (suggesting opposite roles of some PKC isoforms), recombinant stable overexpression of PKCα or PKCδ decreased proliferation capacity and increased the expression levels of differentiation markers; in addition, the overexpressing cells formed smaller and more differentiated tumors. In contrast, the overexpression of cPKCβ or nPKCε had opposite effects: stimulated proliferation and (more aggressive) tumor growth, and inhibited differentiation. Our findings, therefore, strongly argue for pivotal yet antagonistic roles of certain PKC isozymes in the regulation of cellular proliferation and differentiation in human HaCaT keratinocytes.

0196

Invasive phenotype of tumorigenic keratinocytes (SKv) correlates with their constitutional expression of MMP-9 and -2

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Four SKv lines, harbouring integrated copies of the HPV16 genome are derived from the same tumoral lesion of bowenoid papulosis and show either an invasive phenotype (SKv e1, l1) or follow a localised, growth (SKv e2, l2) when inoculated in mice. As organotypic cultures represent an interesting model for studies on skin cancers, we have used SKv lines to reproduce in vitro intradermal invasion of carcinomas in the absence of influences from supplementary cellular elements, e.g.: fibroblasts. The cells were seeded on de-epidermised and devitalised human dermis (DED) and cultured for 1 to 4 weeks at the air/medium interface. Every week, the cultures were examined histologically and immunohistochemically. The culture supernatants were harvested for zymographic, ELISA and Western blot analysis of matrix metalloproteinases (MMP) 2 & 9. In four series of experiments performed on DED from different donors, we have obtained an active penetration through the basement membranes of SKv e1, but not of the other SKv lines, in the presence of 1% of foetal calf serum in the medium. The invasion of the dermis and in vitro vertical migration through porous membranes were absent when the serum was totally substituted by Prolifix of vegetal origin. MMP-9 and MMP-2 were overexpressed by the invasive SKv type, both on DED and in immersed cultures without serum. Active forms of the enzymes were observed with zymography but were not measurable with ELISA. Our study indicates that SKv e1 can actively degrade basement membranes in the absence of other cell types. This rare phenomenon may be exploited for comparative studies of cancers reconstructed with human tumoral keratinocytes originating from the same biopsy but behaving differently, in a model comprising only one type of cells.

0197

The involvement in keratinocyte differentiation of the mitogen-activated protein kinase pathway terminating in extracellular signal-regulated kinase-1/2

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We have previously demonstrated that the protein kinase C (PKC)-activating phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA) activates phospholipase D (PLD) in primary mouse epidermal keratinocytes. Furthermore, we showed that inhibiting PLD-mediated lipid signal production inhibited TPA-induced transglutaminase activity. This result suggested a role for PLD in keratinocyte differentiation independent of the activation of classical or novel protein kinase C isoforms by PLD-generated lipid second messengers. Data in the literature suggest that one product of PLD activity, phosphatidic acid, can serve to recruit Raf-1 to the membrane, where it can be activated and initiate the mitogen-activated protein kinase cascade that culminates in activation of extracellular signal-regulated kinase-1/2 (ERK-1/2). This pathway has been implicated in the proliferative response of keratinocytes to ligation of integrins. However, we found that inhibition of ERK-1/2 activation inhibited TPA-induced transglutaminase activity, a marker of keratinocyte differentiation. Nevertheless, inhibition of PLD-mediated signal generation had no effect on TPA-elicited ERK-1/2 activity nor did inhibition of ERK-1/2 affect PLD activation, suggesting that these two pathways operate in parallel. Indeed, inhibition of the two pathways produced additive effects. Finally, inhibition of ERK-1/2 activation also inhibited transglutaminase activity stimulated by elevated extracellular calcium levels or 1,25-dihydroxyvitamin D. Thus, these results suggest an involvement of both the PLD and ERK-1/2 signaling pathways in mediating keratinocyte differentiation independent of one another.

0198

Over-expression of RhoC GTPase significantly increases Akt phosphorylation in a human melanoma cell line WM1617

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RhoC GTPase is a transforming oncogene for human mammary epithelial cells and can lead to highly invasive phenotype, akin to that seen in inflammatory breast cancer. The gene profiling approach identified RhoC as one of the genes that enhance metastasis in human melanoma cell line A375, and mouse B16 melanoma cell line. Over-expression of RhoC correlates with progression of ductal adenocarcinoma of the pancreas and has been shown to modulate the induction of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and interleukin-8 (IL-8) in breast cells. To clarify the contribution of RhoC in human melanoma progression, we have established stable transfectants over-expressing RhoC, using human melanoma cell lines representing distinct stages in melanoma progression. We investigated whether over-expression of RhoC affects PI3K and NF-kappaB activity in these cells, since these two signaling pathways are important to cell survival, and might be associated with melanoma progression. Western blot showed that over-expressing of RhoC in a human metastatic cell line, WM1617, up-regulated phosphorylated Akt (pAkt) compared with the control. However, expression of Bcl-2 and PCNA was not significantly changed. We also used a dual luciferase reporter assay to assess NF-kappaB activity in these cells and found that RhoC does not enhance NF-kappaB activity in these cells. We are investigating whether this up-regulation of pAkt affects cell proliferation, apoptosis and other characteristics related to melanoma progression in these and other melanoma cells. In summary, over-expression of RhoC activated Akt, but not NF-kappaB in these cells.

0199

Induction of cutaneous melanoma in human skin by growth factors and UVB is dependent on age

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We have shown recently that a combination of three growth factors - basic fibroblast growth factor (bFGF), stem cell factor (SCF) and endothelin-3 (ET-3) - together with exposure to UVB can induce invasive melanoma in neonatal human skin within four weeks. We now investigated if melanocytes can also be transformed in skin from adults. Thirty-eight human skin samples from the abdomen, breast or face from 11 different adult donors were grafted to severe combined immunodeficiency disease (SCID) mice. Increased cutaneous expression of bFGF, SCF and ET-3 in the skin grafts was induced by intradermal injections of adenoviral vectors (Ad5) containing the respective growth factor genes. Additionally, half of the grafts received 50 mJ/cm2 UVB radiation three times weekly. The combined expression of bFGF, SCF and ET-3 led to the formation of nevi and in situ melanomas within 4 weeks of treatment. Interestingly, this seemed to be independent from the exposure to UVB. However, in contrast to our experience with neonatal skins, the lesions in adult skins were less severe and non-invasive. Our data suggest that melanocytes in young people are more prone to malignant transformation by UVB than in adults. This is in accordance with the recently published transgenic hepatocyte growth factor (HGF) mouse model in which UVB exposure of transgenic neonates led to melanoma development, while irradiation of transgenic mice at older age had no such effect. In summary, these data provide experimental support for the epidemiological observations that sunburn particularly in childhood is associated with an increased risk of melanoma development.

0200

Alpha-difluoromethylornithine (DFMO), a Suicidal Inhibitor of Ornithine Decarboxylase (ODC) Prevents Basal Cell Carcinomas (BCCs) in Ptc+/- Heterozygous Knockout Mice

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UVB is a known risk factor for human nonmelanoma skin cancer including BCCs. Skin exposure to UVB induces ODC, which drives continued proliferation and clonal expansion of initiated cells leading to tumor development. DFMO is an ornithine analog that irreversibly inhibits ODC activity and inhibits tumorigenesis in various organs in murine models. ODC is over-expressed in human and murine BCCs. In this study we assessed the effects of oral administration of DFMO on UVB-induced BCCs in ptc+/- mice. Sixty ptc+/- mice were irradiated with UVB (240 mJ/cm2 3 times/week) for 32 weeks. Thereafter, UVB was discontinued and mice were divided into two groups of 30 animals each whereby approximately the same number of tumors were present in each group. Group-1 animals then received water for the next twenty weeks and Group-2 mice received 1% DFMO in drinking water. Five mice from each group were randomly selected and killed at week 37 and their dorsal skin removed for biochemical, histochemical and immuno-histochemcal assays. The remaining animals were continued on the same protocol and all tumors were recorded weekly. After 52 weeks the experiment was terminated, mice were killed and tissues harvested. Twenty weeks after cessation of UVB irradiation, tumor numbers in Group-1 mice receiving water were increased to 300% above baseline whereas in Group-2 mice, tumor number was reduced by ~35% as compared to controls. The number of BCCs and SCCs in DFMO-treated animals was reduced to ~60% of baseline whereas fibrosarcomas were unaffected. DFMO also reduced UVB-induced overexpression of cell cycle proteins such as cyclins A2, B1 and D1 that drive proliferation and also inhibited the expression of COX-2, a marker of tumor promotion. Our data indicate that DFMO is a potent inhibitor of UVB-induced BCCs and SCCs in ptc+/- mice and that strategies directed at inhibiting ODC could be useful for cancer chemoprevention in human populations.

0201

Comparative microarray analysis of the patch and plaque stages of Mycosis Fungoides: implicating a role for oxidative stress?

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Mycosis Fungoides (MF) is the most common of a heterogeneous group of malignant T-cell lymphomas, formally known as Cutaneous T Cell Lymphoma. MF is traditionally thought of as a clonal CD4+ memory T cell disorder primarily manifesting in the skin. MF has a variable presentation with overlap between clinical stages and in its initial stages, is difficult to distinguish from other inflammatory diseases. In the present study, we sought to delineate genomic differences between initial presentation and later disease. We hypothesized that developing unique gene expression profiles for each clinical stage of this malignancy could directly aid in diagnostics, while providing insight into overall disease classification, prognosis and pathogenic mechanisms underlying this disorder. We utilized cDNA microarray technology to construct gene expression profiles from skin biopsies taken from patch and plaque stage MF patients. A 1717 cDNA microarray containing characterized human genes relevant to immunity and cancer was used for differential gene expression analysis of patch versus plaque MF lesions. The data obtained was subjected a non-parametric, nested analysis, which selected 20 genes as potentially differentially expressed. Among the candidate genes identified was an anti-oxidant protein, a member of the RAS oncogene family, and a xenobiotic-metabolizing gene involved in the metabolism of carcinogens. Susceptibility to cancer is frequently a pathological consequence of extensive oxyradical damage that leads to a cycle of cell death and regeneration and causes mutations in cancer-related genes. This data could suggest a role for oxidative stress and oxyradical overload in the development and pathogenesis of MF, as observed in other types of tumors.

0202

Evidence that the induction of growth arrest in malignant melanoma by peroxisome proliferator-activated receptor (PPAR) γ agonists is partly independent of activation of PPARγ

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Peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear receptor superfamily and believed to act as a heterodimer with retinoid X receptor (RXR). Recently, we demonstrated expression of PPARγ in benign melanocytic naevi, primary cutaneous melanomas, melanoma metastases as well as in melanoma cell lines. We also showed that different PPARγ-specific agonists including the thiazolidindione derivates troglitazone and rosiglitazone dose-dependently inhibited cell proliferation in melanoma cell lines. The induction of growth arrest appeared to be through withdrawal from the cell cycle, but was independent from apoptosis. In support of this finding, troglitazone was found to downregulate the expression of cyclin D1 associated with a reduction of detectable hyperphosphorylated retinoblastoma (Rb) gene product. Several experiments were performed with the aim to further dissect the mechanisms involved in the growth inhibition induced by of PPARγ agonists. First, we found that the sensitivity of melanoma cell lines towards the anti-proliferative effects of troglitazone correlated with the protein expression levels of PPARγ. Upon stimulation of cell lines with PPARγ agonists, PPARγ translocated from the cytoplasm to the nucleus as monitored by electron microscopy and immunogold labeling. However, pre-incubation of cell lines with the PPARγ-specific antagonist GW9662 did only reverse growth inhibition by approximately 50%. In addition, co-stimulation of melanoma cell lines with PPARg and RXR agonists did not further enhance the effects of PPARg agonists. We conclude that PPARγ agonists may inhibit cell growth in malignant melanoma at least partly independent of the activation of PPARγ.

0203

Expression of proliferating cell nuclear antigen in mouse skin at different time points following ultraviolet B exposure

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Proliferating cell nuclear antigen (PCNA) is involved in DNA replication, recombination, and repair. PCNA is found throughout the basal layer in normal skin, however in malignant skin lesions PCNA has been seen in all layers of the epidermis and dermis. We previously reported that PUVA and UVB exposure diminished the expression of PCNA in mouse skin. The aim of this study is to evaluate PCNA expression at different time points following exposure to both acute and chronic levels of UVB. In part I of this study SKH-1 mice (3/group) were sacrificed at the following time points: 6, 12, 24, 48, 72 hrs, and 7 days after exposure to UVB 1.5kj/m2. In Part II of this study mice were sacrificed at the time points: 6, 12, and 24 hrs after chronic UVB exposure of 3kj/m2. Immunohistochemistry was performed on paraffin skin sections with anti-PCNA antibody. PCNA expression was quantitated by measuring the number of positively stained cells per 100 keratinocytes. In Part I (1.5kJ/m2 UVB), the results showed the usual pattern of PCNA throughout the basal layer of normal skin. Significantly diminished expression was seen in 6hrs, subsequent time points, 12 hrs to 7 days, PCNA positive cells were progressively increased with pagetoid distribution in all layers of the epidermis. The diminished PCNA expression at the 6 hrs time point is possibly due to the skin undergoing immediate damage in response to UVB. Progressive reactivation of PCNA expression is a result of repair mechanisms initiated by the immune system. In Part II (3kJ/m2 UVB), at 6, 12, and 24 hrs, although there was a progressive increase of PCNA positive cells, the relative number of positive cells were decreased in comparison to Part I. The decrease in the number of PCNA positive stained cells is in response to the chronic levels of UVB. The progressive pagetoid extension of the PCNA cells may indicate a malignant transformation. In conclusion, PCNA can serve as a marker associated with DNA damage and repair, the expression being dose dependent and time related.

0204

Constitutive activation of the ERK1/2 pathway in human melanoma cells, M21 and M24, contributes to their enhanced motility and invasiveness in vitro

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Enhanced migration by melanoma cells is thought to play a critical role in the pathological development of the cancer. The mechanisms, however, remain unclear. We investigated the signal transduction in human melanocyte and melanoma migration and invasion. Using a colloidal gold migration assay with a quantitative computer-assisted analysis (defined as Migration Index or MI), we show here that human melanoma cell lines M21 (primary and invasive) and M24 (primary and metastatic) migrate three to ten times better than parental melanocytes on all major extracellular matrices (ECMs) used. We found that, among the three major MAPK cascades (ERK1/2, p38 and JNK), the ERK1/2 pathway was constitutively activated in both M21 and M24 cells, but not in melanocytes. Further, constitutive activation of ras and Mek1 was also detected in M21 and M24, suggesting that the activating signal initiates from an upstream activator in the plasma membrane. To study the role of constitutive activation of the ERK1/2 pathway in M21 and M24 migration and invasion, we blocked Mek1 signaling by infecting the cells with a lentiviral vector carrying a dominant negative Mek1 gene and by using Mek1-specific inhibitors U0126 and PD98059. These cells were then subjected to a colloidal gold migration assay and an invasion assay in Matrigel-coated Boyden chambers. The results showed that blockade of ERK1/2 signaling reduced the melanoma cell migration and invasion by 60-80%. We also found that the Erk1/2 pathway is necessary, but not sufficient for fully mediating the elevated migratory signals in melanomas. Overexpression of a constitutively active Mek1 (Mek-EE) in melanocytes did not confer a similar enhanced motility phenotype upon normal melanocytes. In conclusion, identification of the Ras-Mek1-ERK1/2 pathway as a key control mechanism for melanoma migration and invasion suggests potential targets for drug design and therapy of this cancer.

0205

Protein kinase C epsilon transgenic mice and the development of metastatic squamous cell carcinoma: the role of inflammation

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Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases. In mouse epidermis, PKCε is one of the skin tumor promoter agent 12-O-tetradecanoylphorbol-13-acetate (TPA) receptors. We have previously reported that FVB/N transgenic mice which overexpress PKCε in basal epidermal cells develop papilloma-independent metastatic squamous cell carcinoma (mSCC) elicited by an initiation (7,12-dimethylbenz[a]anthracene)-promotion (TPA) protocol (Cancer Res., 93: 635-643, 2001). The present histological studies were performed to define the role of cell death and proliferation in the mechanisms associated with development of mSCC in PKCε transgenic mice. Dorsal skin samples were fixed for histological examination at 0, 12, 24, 48, 72, and 96 hrs after a single topical application of 5nmol TPA to FVB/N wild type and PKCε transgenic mice. Recruitment of neutrophils into the skin was observed as early as 12 hrs while skin necrosis was observed at 12, 24, and 48hrs after TPA treatment in PKCε transgenic mice. Increase in skin necrosis correlated with increased numbers and size of neutrophil foci found in skin. The epidermis was completely damaged at 48 hr, but epidermal cell regeneration occurred at 72 hrs post TPA treatment in PKC transgenic mice. The regenerated skin was disordered and showed extensive hyperplasia with evidence of hyperkeratosis. However, these histological changes were observed neither in PKCδ overexpressing transgenic nor in wild type mice. Evidence indicates that precursor cells for papillomas reside in the interfollicular epidermis while carcinoma precursor cells reside in the follicular epidermis (Cancer Res., 60:226-229, 2000). The results presented indicate that PKCε overexpression leads to neutrophil-mediated destruction of epidermis followed by epidermal regeneration from follicular epidermal cells. Taken together these results may explain the mechanism of the development of papilloma- independent mSCC in PKCε transgenic mice.

0206

Human melanoma cells exhibit enhanced matrix attachment and cellular motility compared with melanocytes

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The migration of cells on extracellular matrix (ECM) components is a critical process for cancer cell invasion and metastasis into tissues. Human melanoma cells are highly metastatic. The purpose of this study was to compare the ability of human melanoma cells and human melanocytes to attach and migrate on relevant ECM components. We used a well-characterized computer-assisted cell migration assay in which cells are plated on ECMs and allowed to migrate for 14 hours (before the cell doubling time). After migration, the computer sums the migration tracks in each field and calculates the area that the motility tracks consume in each field, a so-called Migration Index (MI). We found that, in the absence of growth factors, melanocytes did not attach or migrate on any of the matrices tested. Under the same conditions, two human melanoma cell lines (M21 and M24) migrated on all the matrices with MIs of 3-6. In the presence of growth factors, melanocytes migrated minimally on the matrices tested. The MIs were: Laminin = 1, Collagen = 3.5, Vitronectin = 7, Fibronectin = 10. In contrast, M21 and M24 exhibited much greater cellular motility on laminin, collagen and fibronectin. The MIs were: Laminin = 10 (for M24), Fibronectin = 18, and Collagen = 25. Interestingly, however, compared with melanocytes, melanoma cells exhibited decreased migration on vitronectin: MI = 4 (compared with 7 for melanocytes). In a standard cell-matrix attachment assay, the human melanoma cells attached to all of the matrices 5 to 10 fold greater than human melanocytes. FACS analysis of integrin receptors showed that melanoma cells had five-fold the number of $\alpha 3\beta 1$ integrin receptors compared with melanocytes. This may explain why melanoma cells are capable of attaching to and migrating on laminin, while melanocytes cannot. In conclusion, melanoma cells exhibit attachment and migratory properties on ECMs that are distinct from melanocytes. These properties may relate to their metastatic and invasive phenotype.

0207

Immunohistochemical evaluation of lycopene's protective effect against ultraviolet B (UVB)-induced photodamage *in vivo*

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Lycopene is a known potent antioxidant found in tomatoes, and its anticancer properties against certain types of cancers have been well documented in the scientific literature. However, only few investigations explore lycopene's potential role against UVB-induced skin damage *in vivo*. During the process of photocarcinogenesis, UVB radiation is capable of inducing oxidative DNA damage, inducing DNA photoproducts, and promoting the expression of early response genes. The goal of our study was to examine if topical lycopene has a potential protective effect against UVB-induced carcinogenesis *in vivo*, at the level of initiation and promotion. One hour prior to UVB (3 kJ/m^2 at two minimal erythema dose) exposure, female SKH-1 mice were topically treated with two different doses of lycopene (0.05 and 0.1 µmol). Mice were sacrificed 24 hours post UVB irradiation, and immunohistochemical staining was performed for the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG), <6-4> dipyrimidine photoproducts (<6-4> DPs), c-fos, and c-jun. The changes caused by the UVB exposure was expressed by the significantly increased number of positively stained cells for 8-OHdG, <6-4> DPs, c-fos, and c-jun in the positive control, and there were no stained cells for these markers in unexposed skin. Topical application of lycopene reversed and inhibited the damaging effects of UVB radiation, and immunostaining revealed marked reduction in the number of positively stained cells for the aforementioned markers in the lycopene plus UVB treated group. These results suggest, that the naturally occurring carotenoid lycopene may have a protective effect against photocarcinogenesis *in vivo*, by interfering at the level of initiation and promotion.

0208

p53 stabilization by CP-31398 restores wild type p53-dependent function in ultraviolet B (UVB)-irradiated murine skin and in A431 cells

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Mutations in the tumor suppressor gene p53 are detectable in more than 50% of all human malignancies including those affecting skin. p53 is essental for maintaining genetic stability following genotoxic stresses induced by DNA damaging agents such as UVB. Mutant p53 protein is incapable of transactivating its downstream target genes required for DNA repair or apoptosis. CP-31398 is a small molecular weight styrylquinazoline that restores wild-type DNA-binding conformation to mutant p53 protein and re-establishes tumor suppressor functions in vivo. In this study we assessed the effects of parenteral administration of CP-31398 on p53 and p53-dependent downstream target proteins in UVB-irradiated skin of ptc+/- and SKH-1 hairless mice, well-defined models for BCCs and SCCs, respectively. Chronic exposure of ptc+/- and SKH-1 mice to UVB was carcinogenic and led to p53 mutations in tumors and in skin adjacent to tumors. CP-31398 (50 mg/kg/12hr, i.p.) restored the wild-type p53 in skin of UVB-irradiated (240mJ/cm2, single exposure) ptc+/- mice. In addition, there was augmented expression of p53-responsive genes such as p21 and mdm2. Furthermore, CP-31398 reduced UVB-induced overexpression of Bcl-2, PCNA and cyclin D1 but augmented proapoptotic Bcl-XS expression. To further validate the effects of this agent on mutant p53, SKH-1 mice that were chronically irradiated with UVB were treated with CP-31398. This resulted in a marked increase in wild-type p53 as well as up-regulation of p21 and Bcl-XS, and a significant down-regulation of Bcl-2. Furthermore, treatment of A431 cells carrying mutant p53 with CP-31398 resulted in a dose-dependent induction of p21 and Bax and diminution of cyclin D1 overexpression. We also observed an increase in Cytochrome C release followed by activation of caspase 3, associated with G1 arrest and accumulation of cells in sub-G1. These results indicate that CP-31398 restores p53-dependent cell-cycle arrest and apoptosis in skin by stabilizing mutant p53 protein.

0209

Role of prostaglandin E_2 receptors in the pathogenesis of ultraviolet B (UVB)-induced non-melanoma skin cancers

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Enhanced prostaglandin production via cyclooxygenase-2 (COX-2) upregulation is a likely contributing factor in UV-induced non-melanoma skin cancer (NMSC). Prostaglandin E_2 (PGE_2) is the major prostaglandin present in the skin and is increased following exposure to UVB. PGE_2 has four cellular receptors, designated EP1, EP2, EP3, and EP4. This study investigated the expression of PGE_2 receptors in the murine SKH-1 model of SCC and *ptc*+/- model of basal cell carcinoma (BCC). All mice were subjected to a standard complete UVB carcinogenesis protocol and EP expression monitored throughout. The EP1 prostaglandin receptor was expressed at low levels in normal SKH-1 epidermis, but upregulated in UVB-induced hyperplastic skin, benign papillomas, and SCCs. EP1 receptor expression increased as early as 8 hours after a single UVB exposure and persisted for at least 72 hours. EP2 receptor expression was patchy in normal and UVB-irradiated SKH-1 epidermis, with variable expression in UVB-induced SCCs. Patchy suprabasal EP3 receptor expression was present in SKH-1 normal and chronic UVB-exposed epidermis, with moderate expression in some UVB-induced papillomas and SCCs. The EP4 receptor was expressed at low levels in normal and chronic UVB-exposed SKH-1 epidermis but at high levels in sebaceous glands, with variable expression in UVB-induced papillomas and SCCs. In *ptc*+/- mice, all four EP receptors were detected in normal and chronic UVB-exposed epidermis, but no EP receptor expression was detected in UVB-induced BCCs. These findings demonstrate that the EP1 receptor, in contrast to the other EP receptors, is induced by chronic UVB exposure in SKH-1 skin and is upregulated in UVB-induced hyperplastic skin, papillomas, and SCCs, but not in *ptc*+/- murine UVB-induced BCCs. Our data suggest that the increased susceptibility to UVB-induced SCCs associated with overexpression of COX-2 may act through augmented PGE_2 signaling via enhanced EP1 receptor expression. This pathway does not appear to be involved in the pathogenesis of BCCs.

0210

The effects of 12-O-tetradecanoylphorbol-13-acetate, a tumor-promoting agent, and calcium on protein kinase D in primary keratinocytes

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In order to investigate the role of protein kinase D (PKD) in keratinocyte proliferation and differentitation, we studied the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor-promoting agent, and calcium, a differentiating agent, on PKD message levels, protein levels, and activity. We performed a time-point analysis by treating primary keratinocytes for 1, 8, 16, 24 and 48 hours with 100 nM TPA and for 8, 16 and 24 hours with 1 mM calcium. We isolated total protein and performed western analysis using two primary antibodies: one to detect total PKD levels, regardless of phosphorylation status, and one to detect PKD phosphorylated at the serine 916 residue, an indirect measure of kinase activation. We quantified protein levels using enhanced chemiflourescence analysis. We discovered that PKD activity and protein levels, to a lesser degree, were affected in a biphasic manner in response to TPA with an initial increase followed by a decrease and finally a recovery to control levels. This effect on PKD mirrors the acute biphasic response of TPA in keratinocytes in vitro and in epidermis in vivo, where there is an initial stimulation of differentiation followed by proliferation and/or tumor formation. Conversely, the differentiating agent, calcium, caused a decrease in PKD activity and protein levels when compared to control. We are currently isolating total RNA with Trizol at different time points for northern blot analysis. Together, these results suggest that PKD activity corresponds with proliferation and that PKD levels and activity are downregulated during differentiation. Thus, PKD may be an important molecule in terms of possible targets of TPA tumor-promoting activity.

0211

DNA damage and cell cycle alterations in ultraviolet B-induced murine and human basal cell carcinomas

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Aberrant activation of sonic hedgehog characterizes BCCs and mutations in p53 and patched (PTCH) are a feature of UVB-induced murine BCCs and sporadic human BCCs. p53 and ptch are both tumor suppressor genes and are crucial regulators of cell cycle responses to mutagenic agents such as UVB that could explain its carcinogenicity. In this study we tested the hypothesis that UVB-induced BCCs develop because of cell cycle alterations in epidermal keratinocytes carrying mutant ptch. DNA damage checkpoint responses involved in its repair were assessed. Phosphorylation-dependent activation of cell cycle checkpoint kinases Chk1 and Chk2 was detected in both murine and human BCCs, which correlated with enhancement of Chk1- and Chk2-associated kinase activities. Chk1 and Chk2 phosphorylate p53 in vitro at serine 20, a site important for p53 stabilization. Phosphorylation of serine 15 also occurs in response to DNA damage. p53 phosphorylation was confirmed at serines 15 and 20 suggesting that DNA-damage checkpoint responses are active in both UVB-induced murine and sporadic human BCCs. Cdc25C phosphatase which is regulated by Chk1 and Chk2 was overexpressed in BCCs and showed both cytoplasmic and nuclear localization by immunohistochemical staining.We also analyzed the expression of cyclins and their partner kinases related to G1 and S cell cycle transitions and observed up-regulation of cyclins D1 and E as well as cdk4 and cdk6 in both human and murine BCCs. Furthermore the kinase activity of the cyclinB1-cdc2 complex which is crucial for regulation of the G2/M transition was increased in murine and human BCCs. These results indicate that UVB produces cell cycle alterations important for the pathogenesis of human and murine BCCs and reiterate the relevance of the ptc+/- mouse model for human BCCs.

0212

Characterization of porokeratosis by microarray analysis demonstrates similarities with psoriasis not with squamous cell carcinoma

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Porokeratosis is a disorder of keratinization characterized clinically by annular plaques demonstrating a well-demarcated, raised hyperkeratotic border. At the cellular level, porokeratosis demonstrates a clonal proliferation of keratinocytes exhibiting abnormal differentiation; the abnormal clone forms the cornoid lamella, a stack of parakeratin, which is a histologic hallmark of the disease. Although porokeratotic lesions can progress to squamous cell carcinoma (SCC), many do not. In addition, if progression to SCC occurs, the transformation may take years. Therefore, lesions of porokeratosis contain clonally altered cells with an increased propsensity for neoplastic transformation relative to unremarkable skin. To better understand the pathophysiology of porokeratosis, we dissected the cornoid lamella from a porokeratotic lesion, isolated the total RNA, and performed microarray analysis. Surrounding unremarkable epidermis from the same lesion served as a control. We conducted parallel studies on psoriatic lesions and SCC. Our results demonstrate much greater similarity between genes expressed in porokeratosis and psoriasis than between porokeratosis and SCC. Relative to unremarkable skin, genes including calgranulin A and B, psoriasin 1, keratin 6, and keratin 16 were upregulated in porokeratosis and psoriasis but not in SCC. To confirm the validity of our microarray analysis, immunohistochemistry for keratin 16 was performed. In porokeratotic lesions, keratin 16 is highly expressed in keratinocytes forming the cornoid lamella but not in the adjacent keratinocytes. The results of our study demonstrate that there is significant similarity in the gene expression patterns of porokeratosis and psoriasis.

0213

Biochemical activity of Trim32, a cell survival gene associated with carcinogenesis

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We have used a multistage chemical keratinocyte carcinogenesis model to analyze the genetic events leading to initiation and progression of skin carcinogenesis. Elevated expression of Trim32, a member of the tripartite motif family was detected in several, independently derived initiated and tumorigenic cell lines. Elevated Trim32 expression also occurred in all UVB-induced mouse skin tumors tested, but in less than 25% of chemically induced tumors. Furthermore, forced expression of Trim32 in a non-transformed mouse keratinocyte cell strain increased in vitro carcinogenic initiation. Trim32 contains an amino terminal RING zinc finger domain, characteristic of proteins with E3-ubiquitin ligase activity, and a carboxy terminal NHL domain, which mediates protein-protein interactions. Using GFP-Trim32 fusion proteins, Trim32 localized to the cytoplasm, forming a uniform punctuate pattern dependent on the RING domain. This is consistent with the reported function of the RING domain in the formation of supramolecular structures. To understand the biochemical functions of Trim32, a yeast two-hybrid approach was used to identify Trim32 interacting partners. Piasg, the mouse homologue of human PIASy, interacted with Trim32 both in yeast and in mammalian. PIASy is an E3-sumo conjugating enzyme, which inhibits the activity of several transcription factors (including Lef1, Stat1 and p53) through sumoylation. GFP-Piasg protein showed an intense nuclear fluorescence and weak diffuse cytoplasmic fluorescence, consistent with the presence of both nuclear import and export signals in the Piasg protein. We have analyzed the possible ubiquitylation activity of Trim32, and its effects on Piasg stability. We hypothesize that Trim32 may reduce the stability of Piasg protein as it shuttles into the cytoplasm, thus antagonizing Piasg modulation of transcription factors and cell growth and survival.

0214

Inducible activation of a p53 R172H allele accelerates mouse skin carcinogenesis

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Most of the p53 mutations found in human tumors are missense mutations that result in the expression of altered forms of p53. Previous mouse models overexpressing the mutant, p53 R172H, in the epidermis suggested that this mutant p53 promotes carcinogenesis through gain-of-function activities. Since the p53 R172H transgene was expressed at levels 4-8 fold higher than wild type p53, it was unclear whether p53 R172H functions as a true gain-of-function mutation in the genetic sense. To ultimately address this question we have generated transgenic mice expressing one p53 R172H allele in the presence of a wild type allele exclusively in the epidermis. The p53 R172H allele remains silent until activation by an inducible Cre recombinase placed under the control of the keratin 5 promoter. After topical application of an inducer (RU486) Cre is activated, which results in focal activation (removal of a floxed stop cassette) of the p53 R172H allele in epidermal stem cells. Mice expressing the p53 R172H allele were subjected to a skin carcinogenesis protocol. These mice develop tumors faster than control wild type littermates. In addition, the rate of progression to malignant carcinoma was also dramatically accelerated in mice expressing the p53 R172H allele (70% conversion of p53 R172H allele tumors *vs.* 0% of wild type control tumors; 32 wk. after initiating promotion). Furthermore, carcinomas arising in mice that express the mutant p53 R172H allele were shown to metastasize to lymph nodes and lungs. Immunological staining confirmed that mutant p53 was expressed in the epithelial component of tumors at all stages of tumor development and progression. This model provides the strongest *in vivo* data to date that the p53 R172H allele exhibits a true gain-of-function at the genetic level.

0215

Benzo[a]pyrene metabolites enhance the formation of reactive oxygen species and DNA oxidative damage in UVA-irradiated calf-thymus DNA

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Benzo[a]pyrene (BaP) metabolites are capable of inducing reactive oxygen species (ROS) and BPDE-DNA adducts, an initial event in the process of carcinogenesis. UV light activates ROS that contribute significantly to carcinogenesis by inducing oxidative DNA damage primarily represented in these studies as elevated 8-hydroxy-2'-deoxyguanosine (8-OHdG). We reported previously, benzo[a]pyrene and UVA synergistically induce oxidative DNA damage based on considerably increased levels of 8-OHdG. The levels of 8-OHdG in human epidermoid carcinoma cells are much higher than in calf thymus DNA, suggests the metabolized benzo[a]pyrene (in vivo) activates more effectively with UVA. In this study, BaP and BaP-formed metabolites are studied by using UV spectroscopy and HPLC quantitation of 8-OHdG. To determine the specific ROS that participate in the UVA-induced oxidative DNA damage, various ROS quenchers including sodium azide (NaN3), catalase, mannitol and superoxide dismutase (SOD), were studied. Among selected quenchers, NaN3 and SOD were found to inhibit 8-OHdG formation. However, NaN3 inhibited 8-OHdG to a much greater extent than SOD. In addition, NaN3 was found to be the most effective ROS quencher which dose-dependently inhibited 8-OHdG formation by suppressing singlet oxygen generation. We also found BaP metabolites induced 3-folds the levels of 8-OHdG when compared with BaP alone. Therefore, BaP metabolites induce singlet oxygen by contributing substantially to DNA oxidative damage, which differ from superoxide anion, the most contributing agent in parent BaP. Among benzopyrene metabolites, BPDE and BaP-7,8-dione were studied. The data showed that BPDE when irradiated by UVA at small concentrations can induced approximately 10 folds of 8-OHdG than the parent benzopyrene. BaP-7,8-dione did not induce significant oxidative DNA damage when compared to parent BaP. In conclusion, BaP metabolites are more potent in generating ROS and 8-OHdG than parent BaP alone, indicating more potential role in carcinogenesis.

0216

The synergistic carcinogenic effects of benzo(a)pyrene with ultraviolet radiation: models & mechanisms

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Human skin cancer incidence has alarmingly increased over the past twenty years. It has been established that ultraviolet (UV) light is a major cause for skin cancers. Although there has been increased awareness and use of sunscreens, skin cancer incidence is still inclining. Hence, further investigation is necessary to better understand the causes of skin cancer. Humans, apart being exposed to UV rays, and are constantly exposed to environmental pollutants. It has been reported that UVA in combination with certain chemicals in the atmosphere can produce toxic substances that affect human health. We previously reported that oxidative DNA damage was significantly increased by BaP combination with UVA, than UVB. Further we have also reported that the combination of exposures to benzo(a)pyrene and UVA synergistically increased the risk of DNA damage *in vivo*. BPDE adducts, p53 and 8-OHdG levels were increased over time, in the mice exposed to BaP plus UVA. In the current study we further more investigated the carcinogenic effects of synergistic BaP and UVA exposure in SKH-1 hairless mice. Mice were divided into 4 groups of 20 following, Untreated, BaP alone, UVA alone and BaP plus UVA. Mice were treated for 3 times a week/ 25 weeks with the average doses of 2 μg BaP, followed 2 hours later by 40 kJ/m^2 UVA irradiation. From weeks 25-30 mice were only observed while noting the multiplicity of tumors. The results showed that BaP alone or UVA alone did not induce tumors. However, by the end of the 30 weeks, in the group exposed to BaP plus UVA, approximately 70 % of the mice had developed skin tumors with an average multiplicity of 2.2/ mouse. In addition, this group of mice had developed more skin wrinkles and shown significant morphological and histological skin changes. Our results seem to show that atmospheric pollution in combination with UVA light causes a time dependent accumulation of DNA damage and further can induce the multiplicity and incidence of skin tumors.

0217

Identification of differentially-expressed genes in human basal cell carcinomas by DNA microarrays

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The molecular mechanism of basal cell carcinoma (BCC) development from normal keratinocytes is poorly understood. We have used DNA microarray technology to analyze differential gene expression in cultured BCC versus normal keratinocytes in order to detect genes that may be important in the development of BCC. In addition, we employed microarrays to compare differential gene expression in BCC tissues and normal tissues from the same patients. We found that over 3,000 genes are upregulated in BCC and over 800 genes are downregulated in BCC when compared to same-donor normal skin. BCC demonstrates unique expression patterns of keratins, collagens, matrix metalloproteinases, growth factors, and cytokines. Elucidation of the distinctive BCC gene expression patterns may be valuable to our understanding of BCC development and progression, and these patterns may provide targets for new diagnostic and therapeutic options for patients with this common skin cancer.

0218

Shikonin inhibits proliferation of human melanoma cell lines

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The incidence of human skin cancer in the United States is increasing at an alarming rate. Half of all new malignancies are skin cancers and it is estimated that 20% of Americans will suffer from some form of skin cancer in their lifetime. Melanoma is a potentially lethal skin cancer, with a higher incidence of mortality than basal and squamous cell carcinomas. More than 80% of skin cancer deaths are from melanoma. Due to the lethal nature of melanoma skin cancer, and lack of curative therapy, it is of the utmost importance to investigate new preventative agents targeted against this disease. Shikonin, a derivative of the plant *Erythrohizon Lithospermum*, is a commonly used herbal medicine in China. Pharmacological investigation of Shikonin supports this compound's therapeutic efficacy as a potent anti inflammatory with anti tumor and wound healing properties. The present study investigates the effect of Shikonin on human melanoma cell lines. The melanoma cells were transferred to a 96 well micro plate culture system. After incubation the cells were treated with 9 different concentrations of Shikonin. Cellular viability was quantified by utilizing neutral red assay uptake, and measurment of absorbance with spectrophotometry. Shikonin dose dependently inhibited the proliferation of melanoma cell lines when tested against A101D, A875 and OM431. Furthermore, other melanoma cell lines showed similar inhibitory effects when treated with Shikonin. The mechanism by which Shikonin inhibited the growth and proliferation of malignant melanoma cells is thought to be through induction of apoptosis. In conclusion, Shikonin demonstrates a promising capacity to reduce human melanoma cell line proliferation *in vitro*. With subsequent investigations, Shikonin may serve as a novel chemo preventive agent against human melanoma cancers.

0219

Model system to examine the role of the IGF-1R during the UVB response in human keratinocytes

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The major risk factor associated with the occurrence of non-melanoma skin cancer is exposure to the UVB component of sunlight. Exposure of keratinocytes to UVB directly damages cellular DNA. Dependent on the dose, keratinocytes respond to UVB irradiation by repairing the damaged DNA or undergoing apoptosis. An alternative response to UVB irradiation is stress-induced senescence. Previously, we have shown that stress-induced senescence following UVB irradiation is dependent on the activation of the insulin-like growth factor-1 receptor (IGF-1R). This presents an interesting dichotomy of functions, which depends on the specific cell type and a specific cellular environment, since the IGF-1R serves as a conduit for mitogenic keratinocytes growth prior to UVB exposure. However, following UVB irradiation, the IGF-1R enhances cell survival while inhibiting further cellular proliferation. In order to study the role the IGF-1R plays in the response of keratinocytes to UVB irradiation, we have developed a model system which we can selectively inactivate the function of the IGF-1R. HaCaT cells were transduced with the reverse tet repressor and a cDNA of the IGF-1R which contains an inactivating mutation in the ATP-binding domain of the tyrosine kinase. Expression of this mutant cDNA will dominantly repress the activity of wild-type IGF-1R. Since the function of the IGF-1R is required for normal growth of HaCaT cells, the expression of the dominant-negative IGF-1R mutant can be induced by the addition of doxycyline to the cells. The effect of UVB irradiation can then be determined both in the presence and absence of functional IGF-1R depending on the presence of doxycycline in the culture medium. This model system will be used to dissect the biochemical pathways involved in the response of keratinocytes to UVB irradiation both with active and inactive IGF-1Rs.

0220

Peripheral nerves, vascularization, and tumorigenesis in DsRed2-transfected tumors in a mouse model of cancer pain

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Anatomical and functional relationships between the peripheral nervous system and cancers are poorly understood though recent evidence suggests that physical and biochemical interactions between peripheral nerves and tumor cells may be important to both tumor biology and cancer-associated pain. Introduction of coral-derived red fluorescent protein, DsRed2, into a NCTC-2472 fibrosarcoma line has made it possible to accurately distinguish the tumor from surrounding tissue both in vivo and in immunostained sections of the hindlimb in our mouse model of primary tumor-induced mechanical hyperalgesia (2001, J Neurosci. 21:9355). Like the parental line, implantation of DsRed2-2472 cells into and around the calcaneus bone of the C3H/He mouse produces equivalent tumor growth rate, bone remodeling, and robust mechanical hyperalgesia. 2472-DsRed2 cells were implanted into eighty mice and mechanical hyperalgesia testing (wherein a normally non-noxious von Frey monofilament is applied repeatedly to the plantar surface of each hind paw) was conducted at days 5 and 12 post implantation. We used stereological analysis from confocal data sets of immunostained sections to quantify PGP9.5+ and CGRP+ nerves, and blood vessels (CD31+) in DsRed2-2472 fibrosarcomas near the skin at the site of hyperalgesia testing. Results: 1) over time, tumors in hyperalgesic mice have fewer PGP9.5+ nerves and blood vessels; 2) tumors in hyperalgesic mice are more densely innervated by CGRP+ nerves than non-hyperalgesic mice; and 3) tumors from hyperalgesic mice are less densely vascularized than non-hyperalgesic mice. Given the unique vascularity and innervation of solid tumors we hypothesize that changes in sensory innervation and vascularity of the growing tumor contribute to the progressive tumor-induced hyperalgesia observed in our mouse model of tumor pain.

0221

Odontogenic keratocysts in transgenic mice overexpressing the hedgehog signaling effector Gli2

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Odontogenic keratocysts are found in up to 80% of individuals with Nevoid Basal Cell Carcinoma Syndrome (NBCCS), and like BCCs, they are believed to arise due to deregulated activation of the hedgehog signaling pathway. We previously demonstrated that K5 promoter-driven expression of the hedgehog signaling effector Gli2 is sufficient for formation of BCCs. We now report that K5-Gli2 mice frequently develop odontogenic keratocysts. As they aged, some K5-Gli2 transgenic founders exhibited misaligned incisors while others developed obvious mandibular or maxillary masses. Examination of H&E-stained coronal sections from these mice revealed keratinizing jaw cysts and extensive bone remodeling that were strikingly similar to findings in humans with odontogenic keratocysts. Two mouse lines selected for further study revealed the keratocyst phenotype to be highly-penetrant. In the K5-Gli2#27 line, 100% of mice over 6 months of age (N=18) had keratocysts; in K5-Gli2#995 mice, 91% of mice sacrificed after 7 months of age (N=11) had keratocysts. Progeny from multiple generations, currently up to F5, continued to produce keratocysts. Immunostaining of mouse keratocysts revealed the presence of keratins K5, K10, and K17, a keratin expression profile reported in human odontogenic keratocysts. In younger animals, relatively small cysts were found just lateral to molar roots. This localization is consistent with the proposed origin of keratocysts from the epithelial rests of Malassez, cell clusters that are left behind as much of the epithelial root sheath regresses during late stages of tooth formation. Taken together, our findings reveal a remarkable degree of phenotypic similarity between K5-Gli2 mice and NBCCS patients in terms of defects arising in epithelial cells, strongly supporting the concept that endogenous GLI2 mediates responses to deregulated hedgehog signaling in humans.

0222

Contribution of selectin ligands to eosinophil recruitment into the skin: do eosinophils have a skin homing receptor?

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Selectins mediate the initial adhesion of leukocytes to endothelial cells. Mouse T cells migrate into inflamed skin only when they express ligands for both E- and P-selectin. To understand relative roles of selectins in eosinophil recruitment in inflamed skin, we examined the expression of sialyl-Lewis x (sLex) structures and selectin ligands on eosinophils from patients with atopic dermatitis using whole blood flow cytometry. None of the eosinophils from the blood of patients exreppsed HECA-452 (a lymphocyte receptor for skin homing), 2H5 or CSLEX1 epitopes, and they had little avidity for soluble E-selectin. Moreover, none of the infiltrative eosinophils in the skin lesions of patients expressed any type of sLex structures on the surface. In contrast, blood eosinophils bound to soluble P-selectin. The amount of P-selectin that bound to eosinophils was significantly grater in patients with atopic dermatitis than in healthy donors. PSGL-1 expression between these two groups did not differ. The $\alpha(1, 3)$ fucosyltransferases (FucT) are important in the construction of sLex structures and functional selectin ligands. Eosinophils, unlike neutrophils, expressed a large amount of FucT-IV mRNA, but remarkably less or no FucT-VII mRNA. These data indicate that eosinophil interaction with endothelial P-selectin is far more important than interaction with E-selectin for recruitment into the inflamed skin. None of HECA-452, 2H5, CSLEX1 or FH6 antigens functioned as a specific receptor to promote preferential skin infiltration via adhesion to endothelial E-selectin. FucT-IV in eosinophils may be more relevant to the generation of functional P-selectin ligand than FucT-VII.

0223

Fibroblast α3 nicotinic receptor mediates cutaneous effects of tobacco smoke

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To establish molecular mechanisms of effects of tobacco products on precocious skin aging and impaired wound healing, we studied the cell cycle progression and tissue remodeling function of dermal fibroblasts (DF) in the skin of rats and mice after their exposures for 3 weeks to environmental cigarette smoke or drinking water containing equivalent concentrations of nicotine (Nic), using RT-PCR and immunoblotting. We also exposed human DF to 10 mM Nic for 24 h in the presence or absence of mecamylamine, an antagonist of nicotinic acetylcholine receptors (nAChRs). Nic caused 1.9- to 28-fold increase of mRNA and protein levels of p21, cyclin D1, Ki-67, and PCNA, and 1.7- to 2-fold increase of the apoptosis regulators Bcl-2 and caspase 3. Nic also upregulated expression of collagen type Iα1 and elastin, as well as matrix metalloproteinase-1. Mecamylamine abolished Nic-induced alterations, indicating that they resulted from pharmacologic stimulation of nAChRs. To establish relevance of these findings to a specific nicotinergic pathway, we studied human DF transfected with anti-α3 nAChR antisense oligonucleotides and murine DF from α3 nAChR knockout mice. In both cases, lack of α3 was associated with the alterations in the fibroblast growth and function that were just as opposite to those observed in DF treated with Nic, suggesting that the nicotinic effects on DF were in the most part mediated by α3 nAChR. In addition to α3, the nAChR subunits detected in human DF were α5, α7, β2, and β4. The exposures of DF to Nic altered the relative amounts of each of these subunits, leading to reciprocal changes in ligand-binding kinetics. Thus, some of deleterious effects of tobacco on cutaneous extracellular matrix turnover stem from Nic-induced alterations in the unfolding of the genetically-determined program of growth and tissue remodeling function of DF, as well as alterations in the structure and function of fibroblast nAChRs.

0224

Organizing a laminin-5 matrix: the role of the cytoskeleton

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Laminin-5 is a major structural component of the basement membrane in skin. We have developed a model system to analyze how laminin-5 is organized into the matrix of keratinocytes. In the model, keratinocytes are plated onto surfaces coated with rat laminin-5. Normal keratinocytes rapidly remodel rat laminin-5 into higher order assemblies including rings and arcs as they spread over the coated substrate. These arrays of laminin-5 are assembled in an α3β1 integrin-dependent manner, as indicated in studies using function-perturbing integrin antibodies and keratinocytes derived from α3 integrin-null mice. Moreover, keratinocytes extend filopodia and lamellipodia over the substrate as they remodel the surface-bound laminin-5 suggesting that that these cytoskeletal-rich protrusions are involved in assembling matrix polymer into the ring-like patterns under the cells. To test this we treated keratinocytes with various cytoskeleton-disrupting drugs. Agents that induce microtubule depolymerization have no impact on the ability of cells to organize arrays of laminin-5. However, drugs that disrupt the actin cytoskeleton inhibit laminin-5 matrix assembly in our model. Furthermore, keratinocytes that have been treated with a Rho Kinase inhibitor are incapable of organizing laminin-5 into ring-like arrays, revealing that actomyosin contractility and the formation of stress fibers are necessary for this process. Together, our data indicate that functional cooperativity of integrins, the actin cytoskeleton and small GTPases determines the precise supramolecular assembly of laminin-5 in the matrix of keratinocytes.

0225

Impaired epithelization by keratinocytes on glycated collagen I--possible mechanism for deteriorated wound healing in diabetes mellitus?

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Advanced glycation end products (AGEs), that is, products resulting from accelerated chemical modification of proteins by sugars during hyperglycemia, contribute to the pathogenesis of diabetic complications. Extracellular matrix proteins are prominent targets for nonenzymatic glycation because of their slow turnover rates. The aim of this study was to investigate the influence of nonenzymatic glycation of collagen I on wound epithelization. Experimental wound closure by keratinocytes was dramatically promoted on a native collagen I-coated dish compared to an uncoated dish in vitro. When collagen I was glycated with 33 mM glycolaldehyde for up to 144hs, large amounts of AGEs were detected in glycated collagen I. Glycated-collagen I failed to promote epithelization by keratinocytes. The proliferative capacity of keratinocytes did not differ between native collagen I- and glycated-collagen I-coated dishes. In contrast, the adhesion assay revealed that the ability of keratinocyte-collagen I adhesion profoundly deteriorated in a glycation intensity-dependent manner when collagen I was glycated by glycolaldehyde. Adhesion was mediated by the binding of a2b1 integrin on keratinocytes to its ligand GFOGER peptides on native collagen I. However, the GFOGER peptides, per se, could not be directly glycated by glycolaldehyde treatment. These findings suggest 1) that glycation produced substantial amounts of AGEs in collagen I, 2) that the keratinocytes lost their adhesive, but not proliferative, capacity on glycated-collagen I, and that epithelization was indeed inhibited on glycated-collagen I, and 3) that the GFOGER sequence (a2b1 integrin binding site) of collagen I may be masked by a conformational change in glycated-collagen I without direct glycation of GFOGER motif.

0226

Size control of decorin dermatan sulfate during remodeling of collagen fibrils in healing skin

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Decorin is the dominant dermatan sulfate (DS) proteoglycan distributed on the surface of collagen fibrils in the skin. Non-covalent binding of decorin core protein to type I collagen regulates the rate of fibrillogenesis. However a role of its DS chain in organization of collagen fibrils in the skin remains unclear. In order to investigate temporal changes in decorin DS during inflammation and subsequent healing of the skin, 2,4-dinitrofluorobenzene (DNFB) was applied once to the dorsal skin of a mouse and subsequent changes of decorin DS were investigated. Electron microscopic observation revealed that elongated decorin DS was localized among thin collagen fibrils packed loosely in DNFB-treated skin on day 15. In contrast, decorin DS of normal size was distributed among thick collagen fibrils packed tightly in vehicle-treated skin or DNFB-treated skin on day 35. Molecular size of decorin DS increased on day 15 after DNFB application, but decreased to almost normal size on day 35. Disaccharide composition of DS changed most conspicuous on day 15 and content of IdoA2S-GalNAc4S disaccharide unit was in inverse proportion to weight-average molecular weight of decorin DS ($Y=-0.6X+21.7$, $r^2=0.99$). These results suggest that size and disaccharide composition of decorin DS is modulated coordinately in post-inflammatory healing processes, and that control of the size and composition of decorin DS plays important roles in remodeling of collagen fibrils by regulating interfibrillar space in healing skin.

0227

P-selectin-dependent lung colonization of melanoma cells is targeted by heparin treatment

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Interactions between cancer cells and vascular endothelium profoundly affect the spread of circulating tumor cells. Increasing evidence suggests that heparin may exert therapeutic effects in metastatic disease. Our studies employing the B16 melanoma lung metastasis model revealed that heparin inhibited lung colonization only prior to melanoma cell inoculation, whereas heparin treatment initiated after injection failed to suppress lung metastases. These data indicate that heparin interfered with extravasation and initial cell survival. P-selectin-dependent interactions represent a critical step in cell extravasation from the circulation. We thus hypothesized that heparin may reduce melanoma metastases by inhibition of P-selectin binding to its ligands. Since lung colonization is almost completely prevented in P-selectin-/- (P-/-) mice, we next determined whether P-selectin expression by platelets may affect melanoma metastases. In comparison to wild-type (P+/+) controls, lung metastases formation was markedly suppressed by P-/- bone marrow transplantation into P+/+ mice (by > 65%), suggesting that platelet P-selectin expression contributes to experimental lung colonization. The potential impact of endothelial P-selectin in melanoma rolling was evaluated by intravital microscopy of the murine ear, studying interactions of melanoma cells with vascular endothelium. Compared to P+/+ mice, melanoma rolling was reduced by 80% in P-/- mice. To analyze whether heparin inhibits melanoma rolling in a P-selectin-dependent fashion, we investigated the effect of heparin on melanoma rolling in both P+/+ and P-/- mice. Whereas in P-/- mice rolling remained unaffected by heparin, in P+/+ mice heparin treatment reduced rolling of three different melanoma cell lines in a dose-dependent manner. These data support the assumption that both platelet and endothelial P-selectin are potential targets of heparin action in melanoma metastasis formation.

0228

***In vitro* keratinocyte dissociation assay to evaluate blister-inducing activity of pemphigus IgG autoantibodies**

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Patients with pemphigus vulgaris (PV) have circulating anti-desmoglein3 (Dsg3) IgG autoantibodies which induce blister formation. Indirect immunofluorescence or ELISA, which are currently used to measure titers of circulating IgG autoantibodies, do not necessarily provide scores reflecting the strength of blister-inducing activity. The purpose of this study is to develop an in vitro assay to evaluate pathogenic strength of anti-Dsg3 IgG antibodies in disruption of cell adhesion. As isoforms of Dsg compensate each other in their adhesive function, we used primary cultured normal human keratinocytes (NHK) that have low Dsg2 expression while most of keratinocyte cell lines express high level of Dsg2. We also added exfoliative toxin A which specifically cleaves Dsg1 to the assay. We incubated NHK with various antibodies and obtained a dissociation index by comparing numbers of cell particles after mechanical stress by pipetting. When AK23 mAb, which induced PV phenotype in mice when its hybridoma cells were inoculated, and AK7 mAb, which failed to induce the phenotype, were compared, the dissociation index of AK23 mAb was 47.1 times higher than AK7 mAb. When human sera obtained from patients with PV (n=3) in an active stage and in remission were compared, the dissociation indexes of sera from the active stage were 2.1 to 18.5 times higher than those of sera in remission. This dissociation assay will provide a simple and objective method to measure the disruption of cell adhesion associated with blister-inducing activity of pemphigus autoantibodies and a valuable tool to elucidate the molecular mechanism of blister formation and Dsg-mediated cell-cell adhesion.

0229

Impaired Langerhans cell migration in the hyaluronan synthases-1 and -3 double knockout mice

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Hyaluronan (HA), a large glycosaminoglycan consisting of disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, serves as a structural component of both epidermis and dermis. HA synthesis is catalyzed by HA synthases (HAS)-1, 2, and 3, which differ from each other in catalytic activities and molecular sizes of the final products. We observed recently that: 1) dendritic cells (DC) express HAS1, 2, and 3 mRNA, whereas keratinocytes predominantly express HAS2 and 3 mRNA, 2) DC display newly synthesized HA on the surfaces, 3) DNFB-triggered Langerhans cell (LC) migration can be blocked by a HA inhibitor, Pep-1, and 4) Pep-1 also inhibits DC-dependent T cell activation (*J Exp Med* 192:769; *J Immunol* 169:4322). To study physiological functions of HA more directly, we have created HAS1/3 double knockout (DKO) mice. Unlike the HAS2 KO mice, showing embryonic lethality due to developmental defects in cardiac and vascular systems, HAS1/3 DKO mice were born in a Mendelian fashion and exhibited no apparent abnormality. HAS1/3 DKO mice were comparable to the wild-type (WT) controls in the number or morphology of LC in normal skin. Unlike the WT counterparts showing significantly ($P<0.001$) reduced surface densities of epidermal LC after DNFB application, LC numbers remained unchanged in HAS1/3 DKO mice before (667 ± 73 cells/mm^2) and 24 hr after DNFB treatment (696 ± 84 cells/mm^2). DNFB-induced maturational changes (e.g., elevated IA expression and prolonged dendrites), however, were observed in epidermal LC even in HAS1/3 DKO mice. Bone marrow DC cultures generated from HAS1/3 DKO mice were indistinguishable from WT counterparts in the surface HA expression or the ability to activate allogeneic T cells. Likewise, T cells isolated from HAS1/3 DKO mice showed normal proliferative responsiveness to mitogens. We propose that HAS1 and HAS3 deficiency primarily affects HA synthesis and expression in keratinocytes, thereby inhibiting LC migration from epidermis, but not other LC functions.

0230

There is disassembly of adherens junction with increase in cytosolic β-catenin in psoriatic epidermis

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β-catenin, a component of adherens junction (A-J), regulates cell-cell adhesion and cell proliferation, and is increased in various neoplasias. Psoriatic epidermis shows an increase in proliferation and transepidermal migration of keratinocytes. We asked the question as to whether alterations in A-J may play a role in psoriasis. Skin biopsies from involved and uninvolved areas (5 patients) and normal skin controls were studied by electron microscopy (EM), immunocytochemistry (ICC) and by Western blots. EM revealed an increase in intercellular spaces and a reduction in interdigitated extensions, where A-J are frequently located. There was a reduction in cell membrane β-catenin and an absence of the cortical F-actin cytoskeleton in the mid and lower epidermis. These findings are compatible with disassembly of A-J. Western blots of Triton-100 extracts showed a marked increase in cytosolic β - catenin in involved skin, while E-cadherin was not solubilized. c-Src, a kinase localized in A-J, which is known to phosphorylate β-catenin, was increased in involved skin as shown by ICC and by Western blot. Fyn and EGF-R were not increased. The above findings suggest that disassembly of A-J may play a role in regulating keratinocyte proliferation and migration in psoriatic skin.

0231

Up-regulation of MMP-2 Activation and melanoma cells invasiveness by elastin derived peptides through the 67 kDa elastin binding protein

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The interactions between elastin-derived peptides and the 67 kDa elastin binding protein (EBP), was shown to trigger matrix metalloproteinases (MMPs) expression. Here, we examined whether soluble κ-elastin, obtained from alkaline hydrolysis of insoluble elastin, could influence the expression of MMP-1 and MMP-2, and invasiveness of human melanoma cell lines. Our results showed that plating melanoma cells on a κ-elastin substrate resulted in an increase of MMP-2 and TIMP-2 levels, an overprocessing of MT1-MMP, whereas the expression of MMP-1 and integrin αvβ3 were not altered. As melanoma cells showed a positive immunostaining for EBP, the increase of MMP-2 secretion was reproduced by VGVAPG, a ligand of EBP, and was inhibited by lactose which can dissociate EBP from the cell surface. Including κ-elastin within the collagen gels up-regulated MMP-2 but not MMP-1 activation, associated to a decrease of TIMP-2 levels. As a consequence, melanoma cells invasiveness was enhanced by κ-elastin, and the implication of MMP-2 in this process was confirmed since exogenous supply either of rec. TIMP-2, or BB-94, a broad-spectrum MMP inhibitor, reduced this process. Furthermore, the κ-elastin-enhanced melanoma cells invasiveness was significantly prevented after treatment with a S-Gal antibody which can recognize EBP at the cell surface. Altogether, these data suggest that elastin and/or elastin-derived peptides, could up-regulate MMP-2 activation through interactions with EBP, thus facilitating melanoma cells invasion into the dermis.

0232

The specificity-determining loop of the β4 integrin I/A-like domain regulates laminin-5 binding

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α6β4 integrin nucleates hemidesmosome assembly and stabilizes binding of epidermal cells to laminin-5. Analyses of the crystal structure of β3 integrin suggest that its I/A-like domain plays a role in ligand binding. Moreover, a "specificity-determining loop" (SDL) in the I/A-like domain of β1 integrin has been proposed to regulate ligand binding and connects directly to α subunits. Here we investigated whether an SDL-like motif within the I/A domain of β4 integrin regulates binding to laminin-5 and/or to α6 integrin. We generated point mutations within the SDL region of β4 integrin subunit tagged with green fluorescent protein (GFP-β4SDLmut). We also generated a mutation within the I/A-like domain of the β4 integrin, lying outside the SDL region (GFP-β4I/Amut). We transfected constructs encoding the mutated β4 integrin and GFP-conjugated wild type β4 integrin (GFP-β4wt) into 804G cells, which assemble hemidesmosomes, and human endothelial cells, which lack β4 integrin. In transfected 804G cells, GFP-β4wt and GFP-β4I/Amut protein colocalize with hemidesmosome proteins BP180, BP230 and laminin-5. However, in at least 50% of 804G cells expressing GFP-β4SDLmut, hemidesmosome protein localization is disrupted. In endothelial cells expressing these same transgenes, GFP-β4wt protein and the mutant proteins are expressed at the cell membrane and associate with α6 integrin as indicated by precipitation studies of surface-labeled cells. When transfected endothelial cells are plated onto laminin-5 matrix arrays, GFP-β4wt and GFP-β4I/Amut proteins localize precisely with laminin-5 molecules in the extracellular matrix. In sharp contrast, in endothelial cells expressing GFP-β4SDLmut, mutant protein fails to co-distribute along cell-matrix adhesion sites with laminin-5. These results suggest that the SDL segment of the β4 integrin subunit is not required for the formation of α6β4 integrin heterodimers, but is required for ligand binding and certain signaling from matrix to cells that regulates hemidesmosome assembly.

0233

Laminin 10 colocalizes with laminin 5 in the epidermal basement membrane zone beneath hemidesmosomes

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Laminins comprise a family of over 20 extracellular glycoproteins that are involved in cell-basement membrane zone (BMZ) adhesion. In skin, laminin isoforms are present in the epidermal BMZ, around blood vessels (BVs), nerves and adnexal structures. Recently, specific antibodies have become available that recognize laminin chains. The antibody 4C7 recognizes the terminal globular domain of the laminin α5 chain in laminins 10 (α5, β1, γ1) and 11 (α5, β2, γ1). Laminin 5 (α3, β3, γ2) is a well-studied hemidesmosome (HD)-associated laminin. We therefore compared the α5 chain expression with laminin 5 in normal and diseased skin. Laminin α3 and α5 chains were linearly expressed in the epidermal BMZ, except beneath melanocytes. The α5 chain but not the α3 chain was expressed around BVs in adult skin. Late anagen hair follicles stained with anti-α3 and α5 chain antibodies showed typical expression patterns described for HD antigens. In cultured RAC-11P bladder cells, that assemble HD-like structures *in vitro*, both α3 and α5 chains colocalized to discrete focal areas beneath sub-confluent cells presumed to be HDs. Finally, immunogold electron microscopy localized the α5 terminal globular domain to the lower lamina lucida (n=200 gold particles). The majority (75%) of α5 chain labeling was restricted to beneath HDs, similar to laminin 5 (77% labeling restricted to HDs). Laminin α3 and α5 chain expression was assessed in control (n=4) and patients skin suffering from epidermolysis bullosa (EB, n=11). Laminin α5 chain expression was normal in control and all EB subtypes. This was in contrast to the α3 chain that was reduced or absent in JEB subtypes. These data suggest that the α5 chain exhibits an expression pattern restricted to beneath HDs, similar to the α3 chain of laminin 5, however the stable expression of α5 chains is independent of laminin 5. This further supports the hypothesis that several laminin variants may covalently assemble as a multi-molecular complex that together promotes stable epithelial-BMZ attachment.

0234

Suppression of serine proteinases and matrix metalloproteinases facilitates basement membrane assembly in skin-equivalents and improves grafting efficiency

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The basement membrane (BM), located at the dermal-epidermal junction (DEJ), plays important roles not only in adhesion between epidermis and dermis, but also in controlling skin functions. BM structures such as hemidesmosomes, lamina densa, and anchoring fibrils, are rarely observed at the DEJ of skin-equivalents (SE) prepared by culturing human keratinocytes on contracted type I collagen gel containing human fibroblasts. We have recently demonstrated that a matrix metalloproteinase (MMP) inhibitor, CGS23027A (CGS), enhances BM formation in SE by increasing deposition of BM components, especially type VII collagen, and improving the continuity of laminae densae (Exp. Cell Res., 271, 249-262, 2001). In the present study, we searched for factors other than MMPs that impair basement membrane assembly in SE. We found that urokinase-type plasminogen activators were present in culture medium of SE and that aprotinin, a serine-proteinase inhibitor, or tranexamic acid, a plasmin controller, facilitated deposition of laminin 5 and type IV collagen at the DEJ. The deposition of type VII collagen at the DEJ was much further enhanced by the combination of these agents with CGS. Aprotinin plus CGS increased hemidesmosome numbers, and the density and continuity of laminae densae. The effect of the BM quality of SE on grafting of SE to nude mice was examined. Grafted SEs prepared in the presence of aprotinin plus CGS showed better structure of the DEJ and epidermis than control SE cultured without these inhibitors. These results suggest that plasmin-like serine proteinases other than MMPs may impair the assembly of BM in SE by degrading non-collagenous domains of BM components, and that suppression of MMPs and plasmin-like enzymes would be effective to improve the quality of SEs for grafting and in vitro studies.

0235

Lock and key: calcium-stabilized conformation of desmoglein 1 is necessary for hydrolysis by staphylococcal exfoliative toxin

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The blisters in bullous impetigo are caused by ET, a serine protease that shows exquisite specificity in its cleavage of one peptide bond, at a calcium (Ca) binding site in the loop connecting extracellular domains (EC) 3 and 4, in human and mouse Dsg1, but does not cleave other even closely related molecules. To demonstrate that the site-specificity of ET, compared to the sequence-specificity of related serine proteases, such as trypsin, is dependent on the conformation of Dsg1, we showed that Dsg1 preincubated at 56° or higher was not cleaved by ET, but was cleaved by trypsin. Similarly, Dsg1 pretreated at pH 4.5 was not cleaved. Furthermore, pretreatment of Dsg1 with EDTA or dialysis against buffer without Ca prevented cleavage by ET, but not trypsin, even after Ca repletion. Tryptophan fluorescence and circular dichroism demonstrated an irreversible change in conformation of Dsg1 after Ca depletion. This change in conformation was confirmed by loss of epitopes identified by pemphigus foliaceus sera as shown by ELISA and immunofluorescence adsorption studies. Further analysis revealed that only EC1-3 with 4 amino acids after the cleavage site at the end of EC3, but not EC4-5, were necessary for proteolysis. Dog Dsg1, although highly homologous to human Dsg 1, is not cleaved by ET, but substitution of 121 amino acids around the EC2-3 junction from human Dsg1 allows cleavage, showing that this area, 47 amino acids away from the cleavage site, is critical to maintain the correct conformation for hydrolysis. These data suggest that ET fits onto native Dsg1 amino-terminal to the cleavage site, like a key in a lock, to align with the cleavage site, allowing proteolysis. The crystal structure of ET and inferred crystal structure of Dsg1, from comparison with C-cadherin, shows such a fit is possible. This strict dependence on conformation, rather than amino acid sequence flanking the scissile bond, explains the exquisite hydrolytic specificity of ET for one site in Dsg1.

0236

Fibroblast invasion of a fibrin clot requires syndecan 4

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After tissue injury, fibroblast migration from the peri-wound collagenous stroma into the fibrin-laden wound is critical for granulation tissue formation and subsequent healing. Previously we found that fibroblast transmigration from a collagen matrix into a fibrin matrix required the presence of fibronectin (FN). The integrins, alpha4beta1, alpha5beta1 and alphavbeta3, which are known to ligate FN, were found necessary for clot invasion. Now we have examined another family of cell surface receptors: the proteoglycans (PG). From these studies we found that syndecan 4, a heparin sulfate PG, was required for fibroblast migration into the clot. This conclusion was based on beta-xyloside inhibition of glycanation and specific glycosaminoglycan (GAG) degradation. Syndecan 4, a cell surface receptor, known to bind many cationic growth factors, binds FN at the highly cationic Hep II domain as judged by affinity binding with recombinant Hep II. We also found that platelet-derived growth factor (PDGF), which is required to stimulate fibroblast migration, markedly increased cell levels of Syndecan 4-core protein in a time and dose dependent fashion. Increase gene expression was demonstrated by transcriptional studies. These results demonstrate that Syndecan 4 is essential for fibroblast migration into fibrin clots and that PDGF, the stimulus for migration, induces the production of syndecan 4.

0237

Fibroblast invasion of a fibrin clot requires a novel dermatan sulfate-CD44

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After tissue injury, fibroblast migration from the peri-wound collagenous stroma into the fibrin-laden wound is critical for granulation tissue formation and subsequent healing. Previously we found that fibroblast transmigration from a collagen matrix into a fibrin matrix required the presence of fibronectin (FN). The integrins, alpha4beta1, alpha5beta1 and alphavbeta3, which are known to ligate FN, were found necessary for clot invasion. Now we have examined another family of cell surface receptors: the proteoglycans (PG). From these studies we found that dermatan sulfate (DS) was required for fibroblast migration into the clot. This conclusion was based on beta-xyloside inhibition of glycanation and specific glycosaminoglycan (GAG) degradation. CD44, a cell surface receptor known to bind hyaluronan (HA), not infrequently exists as a PG, decorated with various GAG chains including HS and chondroitin (CS), and as such can bind FN. From neutralizing antibody experiments we found that the CD44 core protein was required for fibroblast invasion of the clot. While resting fibroblasts expressed mostly non-glycanated CD44H core protein, platelet-derived growth factor (PDGF), the stimulus for cell migration, induced glycanation of CD44 with CS and DS, but not HS. Thus, DS-CD44H proteoglycan is essential for fibroblast migration into fibrin clots and PDGF induces the production of CS- and DS-glycanated CD44H.

0238

Engineered "Smart" matrix to replace the corrupt provisional matrix in chronic wounds and wounds of the elderly

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Many alterations occur in soft tissue healing of the elderly. One set of alterations, that includes proliferative senescence, low growth factor generation and meager cell response to growth factors, results in suboptimal cell migration and proliferation. Another set of alterations, that includes robust neutrophil infiltration, increase levels of matrix metalloproteinases (MMPs) and decrease levels of enzyme inhibitors, results in deficient fibronectin (FN) matrix in the wound bed. Since human dermal fibroblasts require FN for migration through a fibrin clot, a deficient FN matrix in wounds of the elderly may greatly compound the intrinsic problems of an aging fibroblast population. Chronic wounds also contain high levels of MMPs and an inadequate FN matrix. To address these problems we designed a "Smart" matrix for wound repair that is composed of the FN molecular domains required for fibroblast migration linked to a backbone matrix in such a way to retard proteolysis. Synthetic peptides and recombinant proteins were used to delineate the molecular domains needed for human fibroblast migration over fibronectin. The 9th and 10th fibronectin type III repeats, which contain RGD and PHSRN synergy cell adhesion sequences, support almost maximal fibroblast adhesion, but not migration of primary dermal fibroblasts. Specific sequences within the heparin domain and the IIICS region are also required for migration. These findings predict and additional data confirm the necessity for the cooperation of multiple integrin and non-integrin receptors for fibroblast migration on fibronectin. The 3 FN domains found necessary and sufficient for optimal human dermal fibroblast migration have been linked to a backbone of intermolecularly cross-linked hyaluronan as a first generation of "Smart" matrix for wounds.

0239

Differential effects of laminin-5 and laminin-10 on human keratinocytes

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Laminins 5 and 10 appear to play different roles in the skin. For example, we recently demonstrated that laminin-10 null mouse skin shows defective morphogenesis but normal dermal-epidermal cohesion, whereas laminin-5 deficient skin shows dysadhesion and normal morphogenesis. To determine the mechanisms responsible for differences of laminin function in the skin, we studied the effects of laminins 5 and 10 in cultured human keratinocytes. We performed attachment studies of laminin-5 null (JEBK) keratinocytes to dishes coated with purified laminins 5 and 10. JEBK showed similar levels of attachment to laminin-5 or laminin-10 under normal conditions, however, after growth factor depletion, JEBK showed significant impairment of short term and long term adhesion to laminin-10 compared to laminin-5. After attachment, JEBK showed increased spreading and flattening when plated on laminin-5 compared to laminin-10. JEBK showed impairment of hemidesmosome formation on laminin-10 compared to laminin-5. JEBK showed inhibition of focal adhesion formation on laminin-10 compared to laminin-5, especially under growth factor starving conditions. Consistent with these results, normal keratinocytes (NK) incubated with laminin-5 inhibitory antibodies showed decreased FAK phosphorylation compared to NK incubated with laminin-10 inhibitory antibodies. Both laminins 5 and 10 appeared to play roles in cell migration. In scratch assays, NK cells showed impairment of migration either in the presence of laminin-5 or laminin-10 inhibitory antibodies. Interestingly, JEBK showed significantly increased migration in scratch assays on laminin-10 compared to laminin-5. Under growth factor depleting conditions, JEBK plated on laminin-10 showed reduced proliferation compared to JEBK plated on laminin-5. In summary, we have shown a number of differences in the effects of laminin-5 and laminin-10 on keratinocytes, which could account for the different roles that these molecules play in the development and function of the skin.

0240

Characterization of the interaction of the bullous pemphigoid antigen 1 (BP230) and desmoplakin with distinct intermediate filament proteins

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BP230 and DP are members of the plakin family of cytolinkers. Despite their high homology, their COOH termini associate with distinct intermediate filaments (IF). We studied sequences within their COOH termini necessary for their interaction with keratins K5/K14, keratins K8/K18, and type III IF vimentin by yeast three-hybrid, cell transfection and overlay assays. We found that BP230 interacts with K5/K14, but not with K8/K18 or vimentin, via a region encompassing both the B and C subdomains and the COOH extremity, including a COOH-terminal 8-amino acid stretch. The C subdomain with the COOH-terminal extremity of DP bind to K5/K14 and K8/K18, while its linker region is able to associate with K8/K18 and vimentin. Results obtained with chimeric constructs in which distinct regions of BP230 were swapped with the equivalent sequences in DP confirmed the importance of the linker and/or the COOH extremity of DP for IF binding. Furthermore, the potential of DP to interact with IF proteins in yeast seems to be regulated by phosphorylation of Ser 2849 within its COOH terminus. Strikingly, BP230 and DP only interacted with cytokeratins when both type I and type II keratins were present, suggesting that the tertiary structure induced by their heterodimerization is critical for their association with plakins. Removal of the head and tail domain from K5 and K14 did not abrogate their binding to DP and BP230. Our findings indicate that 1) sequences within the linker and the COOH extremity of plakins have a critical impact on their binding activity by conferring specificity for various IF proteins ; 2) the association of DP and BP230 with epidermal and simple keratins requires heterodimerization of these IF proteins; and 3) the rod domain of K5/K14 harbors important recognition sites for plakins.

0241

Laminin 10 expression and correlation with squamous cell carcinoma invasiveness

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As it has recently been appreciated that the aggressive behavior of malignant tumors is related to their extracellular environment, the goal of this study was to examine the expression of laminin 10, a newly identified major extracellular matrix component of skin basement membranes, in human squamous cell carcinomas (SCC). Skin samples were collected from 50 patients, including 10 actinic keratosis (AK), 13 SCC in-situ or Bowen's Disease (BD), 19 invasive SCC and 8 normal controls. Frozen sections were examined by immunofluorescent staining with monoclonal antibody 4C7, which is specific for the alpha 5 chain of human laminin 10. A Zeiss Axiovert 200 microscope with a digital camera and image analysis system was used to capture the images. The intensity of staining was quantified and expressed as mean gray value per pixel (square picture element). Statistical differences between groups were determined by one-way analysis of variance. Significant higher expression of laminin 10 was detected in the basement membranes of invasive SCC tumor masses and newly formed tumor blood vessels compared with normal skins. Strikingly, the studies demonstrated incremental expression pattern as tumors progress from pre-malignant lesions of AK to malignant SCC. The mean gray values were 4.11 for normal skin, 8.66 for AK, 9.21 for BD and 14.29 for SCC. $P < 0.001$ when comparing AK, BD, SCC to normal skin and when comparing SCC to AK and BD respectively. In addition, by Western blot and immunofluorescent staining, high expression of laminin 10 was found in cultured SCC cells compared with normal human epidermal keratinocytes. The studies revealed a clear correlation between the expression level of laminin 10 and SCC tumor stage of invasiveness and indicate that laminin 10 play important roles in SCC angiogenesis and invasion. Investigations of animal model and mechanisms involved are underway.

0242

Cellular phosphorylation initiated by pemphigus vulgaris IgG binding to the demosomal cadherin desmoglein 3

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In the human autoimmune blistering disease pemphigus vulgaris (PV), pathogenic antibodies bind to the desmosomal cadherin desmoglein 3 causing suprabasilar epidermal cell-cell detachment (acantholysis). The mechanism by which PV antibodies trigger acantholysis remains unknown; however, proposed mechanisms include (1) proteinase activation, (2) steric hindrance, and (3) activation of transmembrane signaling that down regulates cell-cell adhesion. We sought to investigate the potential of PV IgG to initiate signaling events by disruption of desmosome mediated cell-cell adhesion. Normal human keratinocytes, cultured in the presence of $[^{32}P]$-H_3PO_4, were exposed to PV IgG, control IgG or buffer. Two dimensional gel electrophoresis of ^{32}P-labeled keratinocyte extracts was used to identify changes in protein phosphorylation. Dose and time dependent changes in protein phosphorylation were observed in cells treated with PV IgG. These observations suggest that PV IgG induced changes in desmosomal adhesion likely activate cellular signal transduction cascades and provide support for the hypothesis that transmembrane signaling may have a pathogenic role in PV IgG induced acantholysis.

0243

The effects of keratinocytes and fibroblasts on the formation of basement membrane in skin equivalent models

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The cutaneous basement membrane (BM) plays important functions in normal and pathologic conditions. To investigate the effects of keratinocytes and fibroblasts on the formation of cutaneous BM, 1. human skin keratinocytes were cultured on dead de-epidermized dermis (KC-DED) 2. keratinocytes and fibroblasts on DED (KC+FB-DED) 3. keratinocytes on reticular dermis (KC-dermis) 4. keratinocytes and fibroblasts on reticular dermis (KC+FB-dermis) 5. keratinocytes on collagen gel without fibroblasts (KC-CG) 6. keratinocytes on fibroblast-populated collagen matrix (KC-FBCG) 7. keratinocytes on DED combined with fibroblast-populated collagen matrix (KC-DED+FBCG) in air-liquid interface for 10 days and immunohistochemical and electron microscopic studies were done. In KC-dermis, KC-CG and KC-DED, β 4 integrin, laminin, type 4 and 7 collagen were all expressed at the dermo-epidermal junction. In KC+FB-dermis or KC-FBCG, laminin, type 4 and 7 collagen were expressed more strongly than KC-dermis or KC-CG. In KC+FB-DED and KC-DED+FBCG, type 4 collagen was expressed more strongly than KC-DED. Electron microscopic studies showed that in KC-dermis and KC-CG, formation of BM was rarely seen. In KC-FBCG, BM zone such as hemidesmosomes, lamina lucida, lamina densa and anchoring fibril were formed, although BM was not still continuous. In KC-DED, KC+FB-DED and KC-DED+FBCG, BM zone was found similarly to that in normal skin. Interestingly, in KC+FB-DED some fibroblasts in upper dermis and partial loss of basal lamina around the fibroblasts were seen. These results show that keratinocytes produce β 4 integrin, laminin, type 4 and 7 collagen. Also, fibroblasts stimulate expression of components of BM and formation of BM zone, suggesting that fibroblasts may produce laminin, type 4 and 7 collagen or influence the effects of keratinocytes on the formation of BM.

0244

Aging alternations of N-linked oligosaccharides from dermis

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Oligosaccharides moieties of glycoproteins play important roles for modulation of protein properties as stability and biological activities, and various molecular recognition processes by their structural diversity. There are many evidences that structures and ratios of oligosaccharides are altered in various physiological and pathological events, such as tissue differentiation, cancer, immune response, development, fertilization and aging. However, it has not been understood alternations of skin oligosaccharides. In this study, we obtained dermis from young and aged mice, and compared structures and ratios of N-linked oligosaccharides with two-dimensional sugar mapping technique using pyridylamino labeling. As a result, the ratio of, galactose residues on, biantennary complex type oligosaccharide was decreased in aged dermis. Furthermore the ratio of bisecting N-acetylglucosamine and α1-6fucose residue was increased.

0245

Detail of N-linked oligosaccharides from dermis

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Oligosaccharides of lipids and proteins control cell developments and function as ligands for intercellular adhesion receptors, cell matrix proteins, growth factors, toxin and microbes. Many histochemical studies using lectin and antibodies recognizing saccharide moieties have shown the presence of different cell surface glycoconjugates in skin. However, it has not been clear detail structures of whole oligosaccharides and where the saccharide component binds lipid or protein. In this study, we analyzed detail structures and ratios of N-linked oligosaccharides from murine dermis. Dermis was diegested using trypsin and chymotrypsin. N-linked oligosaccharides were released from glycopeptidase by digestion with N-glycosidase F. The reducing ends of the oligosaccharides were derivertived with 2-aminopyridine. The pyridylamino oligosaccharides were identified by two-dimensional HPLC mapping technique. As a result, we determined 14 kinds of oligosaccharide of dermis. Complex type oligosaccharide was 87.74% of total and high mannose type was 12.26%. And, we makes clear that the most predominant structure is Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc.

0246

Detection of alternative splicing in *COL7A1* : inserting 9 amino acid residues into the fibronectin type III linker domain

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Type VII collagen is the major component of anchoring fibrils within the cutaneous basement membrane zone. The large amino-terminal noncollagenous domain (NC1) interacts with various extracellular matrix (ECM) proteins and contributes to the dermal-epidermal attachment. The purpose of this study was to detect alternative splicing of *COL7A1*transcript encoding the NC1 domain. We also examined expression of the alternative splicing in situations relating wond healing and skin remodeling. Amplification of overlapping cDNAs from keratinocytes using RT-PCR identified alternative splicing, which was generated by a different exon 18 acceptor site 27 bp upstream from the common acceptor site. Expression of this alternatively spliced transcript differed among several cell types. An exon-trapping experiment using a related genomic DNA fragment comfirmed existence of this alternative splicing. The 9 amino acid residues GPLTLPLSP from the 27 bp nucleotides were inserted into the linker of fibronectin type III (FN3) domains. This insertion was suggested to contribute to flexibility of the linker of FN3 domains and may affect the interactions between the NC1 domain and ECM proteins. Treatment with transforming growth factor TGF-β1, which promotes wound healing and skin remodeling, enhanced the expression of this 27bp transcript. Furthermore, keratinocyte biopsies from the wound edge of patients with epithelizing skin ulcers showed a significant increase in the 27bp-transcript expression compared with normal keratinocytes from steady-state body sites. These results suggest that amino acid variation of this alternative splicing may have some role in dermal-epidermal adhesion, wound healing and skin remodeling. To our knowledge, this is the first evidence of alternative splice insertion of a small peptide into the linker region of the FN3 domains, a common motif within modular proteins.

0247

Assessment of splice variant-specific functions of desmocollin 1 in the skin

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Desmocollins (dsc) are thought to be essential for the assembly and function of desmosomes. Each of the three known dsc genes encodes two proteins (a and b-form), the corresponding mRNAs are generated by differential splicing. The a-forms bind several desmosomal plaque-proteins and are thought to be necessary to assemble the plaque and to connect the desmosome to intermediate filaments. No specific function has been associated with the b-forms. To investigate the function of an dsc a-form in-vivo, we have generated mice that are deficient for desmocollin 1a. Surprisingly, homozygous mutant mice showed no abnormal phenotype in terms of birth weight, growth rate, coat development and skin development. Immunofluorescence microscopy of newborn skin with various antibodies against desmosomal proteins and other cytoskeleton markers revealed no obvious abnormalities in epidermal differentiation. Furthermore, the remaining dsc1 isoform was expressed in mutant skin and incorporated into desmosomes, suggesting that dsc1a is not required for the incorporation of dsc1b into desmosomes. However, gene expression profiling indicated several interesting biochemical abnormalities in mutant mice. For example, dsc2 expression was dramatically upregulated, and several hair keratins as well as keratin-associated proteins were down-regulated in these mice. Further studies will have to determine whether subtle ultra-structural abnormalities occur due to these biochemical changes. Finally, dsc1a-/- mice do not show any of the phenotypes that occur in dsc knockout mice, i.e. mice that are deficient for both dsc1 splice variants. Our interpretation of these results is that dsc1a is not required to establish and maintain desmosomal adhesion. However, a minimal amount of dsc1 transmembrane receptors, provided by dsc1b, is necessary to maintain structural integrity of the skin.

0248

The novel MMP-21 is expressed during skin carcinogenesis and induced by TGF-beta1

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We have recently cloned a novel metalloproteinase, MMP-21, from placenta cDNA (Ahokas et al. Gene 2002:301:31). This 57 kDa protein contains a signal sequence, a prodomain, a zinc-binding catalytic domain, and a hemopexin-like domain flanked by two Cys residues, a furin activation sequence but no transmembrane sequence. It is an orthologue for the Xenopus MMP and most closely related to MMP-17, -23 and -25. By Northern analysis, MMP-21 is expressed at low levels during fetal development. By RT-PCR it has been detected in many adult tissues and epithelial cancer cell lines. By immunohistochemistry MMP-21 was found in the epithelium of colon and ovarian cancers. The aim of this study was to investigate the role and regulation of MMP-21 in epithelial cell lines and skin cancer. To study the expression and cellular localization of MMP-21, we used keratinocyte and HaCaT cell cultures, quantitative TaqMan RT-PCR, transfections to 293A cells, and immunohistochemistry on human tissues with our polyclonal antibodies. MMP-21 transfected into 293A cells resulted in cytoplasmic staining. MMP-21 is not expressed by unstimulated primary keratinocytes, but is present in HaCat cells. Of several cytokines relevant in development and skin cancer tested, TGF-beta1 induced MMP-21 in HaCat cells. Analogously, MMP-21 protein was undetectable in normal skin or premalignant conditions such as Bowen's disease. It is not induced by benign keratinocyte hyperproliferation as seen in chronic ulcers or psoriasis nor by migrating phenotype as evident in normally healing wounds. MMP-21 is not detected in basal cell cancer but a subset of squamous cell cancers (Grade III tumors) express MMP-21 in cancer cells at the invading front, suggesting that this metalloproteinase is an important contributor to the invasion of cutaneous squamous cell cancer.

0249

Analysis of Jararhagin, a collagen mimetic substrate, on MMP expression: characterization of functional domains for integrin receptor activation

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Cell matrix interactions play an important role in wound healing, tumor invasion and metastasis. We have shown, that binding of the adhesion molecule integrin $\alpha2\beta1$ to type I collagen initiates several signalling events including the regulation of matrix metalloprotease (MMP) synthesis and activation, in particular of MMP-1 and MMP-14. Due to the function to degrade matrix components these enzymes are crucial for tissue remodeling. Synthesis of MMP-1 and MMP-14 was found to be induced upon treatment of fibroblasts in monolayer culture with Jararhagin, as seen in western and northern blots, which was comparable to that obtained in fibrillar collagen lattices. This snake venom metalloprotease containing a disintegrinlike (Dis) and a metalloprotease (MP) domain binds $\alpha2\beta1$ integrin and induces intracellular signalling events similar to collagen. In addition treatment with Jararhagin leads to a change of cell morphology to a spindle-like phenotype and an inhibition of collagen/integrin interaction. To get more insights in the mechanisms underlying the Jararhagin/integrin interaction we further charcterized signalling by other SVMPs. We tested recombinant Dis and MP domains with high homology to Jararhagins domains. Adhesion assays have shown that the Dis but not the MP domain are bound by fibroblasts. Alterations of cell morphology as observed by Jararhagin were only induced by the MP domain but only in its active state while the Dis domain did not affect the cell shape. Interestingly, both domains were able to independently induce MMP-1 synthesis and MMP-14-mediated pro MMP-2 activation and to interfere with collagen interactions. These data show that both domains are responsible for the inducing effect on MMP synthesis. Moreover, as the Dis but not the MP domain binds to fibroblasts these two domains use different pathways to affect MMP synthesis.

0250

Modulation of skin collagen metabolism by dehydroepiandrosterone in human skin *in vivo*

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Dehydroepiandrosterone (DHEA) and its sulfate conjugate (DHEA-S) are the most abundantly produced adrenal steroids whose levels in human are decreased according to aging. DHEA showed beneficial effects on delaying the onset of diabetes, immune decline, obesity, and tumor. DHEA may be related to the process of skin aging through the regulation of production and degradation of extracelluar matrix. In this study, to investigate the effect of DHEA as an anti-skin aging agent, we have examined whether DHEA can modulate type I procollagen, MMP-1 and TIMP-1 expressions in human skin *in vivo*. 5% DHEA in ethanol:olive oil (1:2) was topically applied to buttock skin of old volunteers (n=6, average 80yrs) 12 times for 4 weeks. Topically applied DHEA significantly increased the expression procollagen mRNA and protein by 117 % ($p<0.05$) and 122% ($p<0.05$) compared with vehicle treated skin, respectively, as determined by RT-PCR and western blot. The expression of TIMP-1 mRNA and protein was increased by 106 % and by 120 % ($p<0.05$), respectively. MMP-1 mRNA expression was decreased by 48%($p<0.01$), and MMP-1 protein tended to decrease. We also studied the time-and dose-dependent effects of DHEA on the expression of type I procollagen, MMP-1 and TIMP-1 mRNA and protein in human dermal fibroblasts. In cultured dermal fibroblast, DHEA(10μM-50μM) increased production of procollagen $\alpha1$ (I) mRNA and protein, compared with control cells. In contrast, the expression of MMP-1 mRNA and protein was decreased at 10μM for 72hr. The expression of TIMP-1 mRNA and protein showed a dose-dependent increase compared with control cells. Treatment of DHEA in the cultured fibroblast suppressed the UV-induced AP-1 binding activity and MMP-1 protein expression. These results strongly suggest the possibility of using DHEA as an anti-skin aging agent of human skin.

0251

Role of novel desmosomal cadherin, desmoglein 1-β, in cell-cell adhesion and blistering diseases

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Desmogleins (Dsg) are transmembrane core components of the cell-cell adhesion structure, the desmosomes, and belong to the cadherin supergene family of calcium-dependent adhesion molecules. In addition to the three known isoforms of Dsgs (Dsg1, Dsg2, and Dsg3), we recently cloned a novel mouse desmoglein gene *(Dsg1-β)* sharing high homology to both mouse *Dsg1 (Dsg1-α)* and human *DSG1*. The *Dsg1-β* gene is 32 kb downstream of the *Dsg1-α* gene and is encoded by 15 exons spanning 45 kb on mouse chromosome 18. Sequence conservation between *Dsg1-α* and *Dsg1-β* (96% identity) diverges significantly at exon 11 (79%) particular in the region that encodes the extracellular anchoring domain. Dsg1-β protein shares 94% and 76% identity with mouse Dsg1-α and human DSG1, respectively. To examine the expression pattern of Dsg1-β, we raised antibodies against Dsg1-β, which did not recognize Dsg1-α. We showed that Dsg1-β was co-expressed with Dsg1-α in the differentiating cell layers of the epidermis. The most striking difference was observed in the eye where Dsg1-α was completely absent while Dsg1-β was highly expressed in the limbal epithelial cells. Although an orthologous DSG1-β gene was not found in the human genome, we detected Dsg1-β expression in the highly differentiated cell layers of the human epidermis of skin. Interestingly, while staining for Dsg1-β was normal in non-striate palmoplantar keratoderma tissue, Dsg1-β expression was disrupted in epidermis of striate palmoplantar keratoderma (S132X) patient with a nonsense mutation in the *DSG1* gene. These results suggest that in human, Dsg1-β maybe an alternatively spliced product of the *DSG1* gene.

0252

New collagen-peptide improves skin structure and promotes cell adhesion, differentiation and extra cellular matrix synthesis

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In the skin, collagen plays a crucial role in maintaining structural integrity, tissue remodeling, and wound healing, and is also very important for cell adhesion and migration. We have developed a synthetic oligo collagen-like peptide and studied its effect on human cultured skin cells and ex vivo models. Cell adhesion assay using HaCaT cells showed that when the cells were seeded in the presence of 0.001 mM of peptide, their adhesion to the microplates was clearly enhanced. Cell migration assay showed no evidence of increased cell migration caused by the peptide. Time course studies and H&E staining of cultured fibroblasts confirmed the role of the peptide in enhancing cell adhesion that led to a nice uniform cell sheet in treated wells. Immunofluorescence studies on subconfluent fibroblasts revealed that treatment of the cells with 0.001 mM of the peptide increased the expression of ECM molecules (such as fibronectin, collagen I and III). Treatment of A431 cells with the peptide enhanced their synthesis of keratin and adhesion molecules such as integrins (alpha 6, beta 1) and laminin 5. Moreover, human skin ex vivo studies showed that skin samples treated with the peptide and cultured for 72 hours, exhibited a remarkably better epidermal and dermal structure than control samples. The epidermis showed no signs of stress, and the dermis had a superior fibrillar structure in the peptide-treated samples. Interestingly, immunofluorscence studies of differentiation markers expression in skin samples, such as filaggrin, showed an increased expression of filaggrin in peptide-treated samples and confirmed that the peptide enhanced epidermal differentiation. These results show that this new collagen-like peptide stimulates ECM biosynthesis and exhibits a great number of collagen-like effects on skin cells. This peptide is therefore of great interest in tissue regeneration and in topical skin care products.

0253

A secretion from *Cryptomphalus aspersa* increases human fibronectin matrix assembly capacity by CHO cells

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SCA is a secretion obtained from the molluscus Criptomphalus aspersa when submitted to controlled stress challenge. Its main components are a non-copper hemocyanin, a fibroblast growth factor "like" protein, antioxidants enzymes (SOD) and hyaluronic acid. A preparation containing SCA has been successfully used in wound repair in radiodermitis showing beneficial skin repair properties. The aim of this study is to evaluate if repairing activity of SCA is related to matrix reorganization capacity. Extracellular matrix play and important role in cellular metabolism and functions, as cells receive information from the exterior through the adhesion receptors present on the cellular membrane. Matrix assembly assay is a new model used to study the effect of active ingredients on matrix organization by studying their effect on the assembly of human fibronectin. We used CHO cells that assemble human fibronectin when an optimal concentration is used. In our study, we determined the pro-assembly activity of SCA in presence of CHO cells when suboptimal concentration of human fibronectin ared used. SCA is negative for fibronectin content as determined by ELISA and Western-blot techniques. CHO cells are incubated overnight in the presence of different concentrations of human fibronectin and different concentrations of SCA. Then, cell were fixed and analyzed by indirect immunofluorescence with antibodies anti-human fibronectin. SCA is able to induce fibronectin assembly by CHO cells in presence of suboptimal concentration of human fibronectin. No assembly is observed in absence of SCA in those experimental conditions. As fibronectin is not present in SCA, this pro-assembly capacity could be due either to integrin activation on CHO cells or by a direct interaction of fibronectin with a cell-interacting SCA component. In conclusion, this proassembly activity may be related to the wound repair effects, which was found in radiodermitis patients.

0254

Shedding of collagen XVII/BP 180: specific structural motifs influence cleavage from cell surface

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Collagen XVII, an epithelial adhesion molecule, belongs to the family of transmembrane collagens. This novel family currently includes seven members with extracellular collagenous C-termini which can be shed from cell surfaces. Shedding of collagen XVII is mediated by membrane-bound ADAMs, which cleave their substrates close to the cell surface. They do not require strict sequence specificity, implicating a structural motif for substrate recognition. Here we analyzed the shedding of collagen XVII, which is cleaved within the juxtamembranous non-collagenous NC16A domain. To localize the sequences required for shedding, COS cells were transfected with cDNA for full-length collagen XVII and different recombinant deletion mutants. The full-length form was expressed and shed efficiently, but mutants with large deletions within the NC16A domain were not. In contrast, deletion of the 18 most N-terminal amino acids within the NC16A domain, enclosing a furin cleavage site, did not prevent shedding, excluding a role for furin as a genuine convertase. Analysis of defined small deletions pointed to amino acids residues 528-547 of collagen XVII as the recognition site for ADAMs. This region spans the putative N-terminus of the 97 kD LABD antigen, a fragment of collagen XVII ectodomain, which is recognized by autoantibodies in linear IgA disease. Secondary protein structure predictions of the deletion mutants revealed that all non-shed mutants shared an NC16A domain with a positive net charge and included one α-helix with a putative amphiphatic element, which can cause conformational changes in the collagen XVII homotrimer. The data indicate that the conformation of the NC16A domain and steric availability of the cleavage site to ADAMs influence the shedding and thereby regulate cell adhesion and motility. Further, mutations in the NC16A domain are likely to change the conformation of the ADAMs recognition site and thus contribute to abnormal shedding of collagen XVII in junctional epidermolysis bullosa.

0255

Dystroglycan in skin and cutaneous cells: beta-subunit is shed from the cell surface

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The hemidesmosome attaches the epidermis to the dermis and is critical for stable connection of the basal epithelial cell cytoskeleton with the basement membrane. Post-translational processing of several molecular components of the adhesion complex, such as collagen XVII, laminin 5 or collagen VII, regulates its functions in keratinocyte adhesion and motility. In muscle, a similar supramolecular aggregate, the dystrophin glycoprotein complex links the inside of muscle cells with the basement membrane. A component of the muscle complex, dystroglycan, a type I transmembrane protein, also occurs in epithelia. Here we characterized the expression and biochemical properties of authentic and recombinant dystroglycan in human skin and cutaneous cells in vitro. In skin, dystroglycan is present at the epidermal basement membrane, and it is produced by both keratinocytes and fibroblasts in vitro. The biosynthetic dystroglycan precursor was efficiently processed to the alpha- and beta-subunits. A special feature of the cutaneous cells was that the extracellular part of the transmembranous beta-subunit was proteolytically shed from the cell surface thus releasing the alpha-subunit into the culture medium. Shedding of the beta-subunit was enhanced by EGF, IL-1 beta and phorbol esters, and inhibited by inhibitors of furin, serine proteinases and metalloproteinases, suggesting that an enzyme cascade is involved in this process. Deficiency of perlecan, a major basement membrane proteoglycan and ligand of alpha-dystroglycan, enhanced shedding, suggesting that lack of a binding partner destabilizes the epithelial dystroglycan complex and renders it prone to cleavage. Taken together, we identified dystroglycan as a novel component of the epidermal basement membrane and extended the spectrum of proteins in this zone that are regulated by post-translational proteolytic processing.

0256

Dermatan sulfate synthesis is stimulated by three-dimensional culture

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Dermatan sulfate (DS) is the predominant glycosaminoglycan (GAG) in the extracellular matrix of skin. It plays key roles in biological processes including development, growth and wound healing by binding and activating growth factors and enzymes such as FGFs and heparin cofactor II. While monolayer or two dimensional (2D) cell culture is a common in vitro model for studying these processes, three dimensional (3D) culture is required for recapitulation of many events observed in vivo. The factors responsible for this phenotypic shift in 3D versus 2D are not known. The purpose of this study was to determine if a change in quantity and composition of GAGs or proteoglycans (PGs) produced by fibroblasts grown in 3D collagen gel culture compared to 2D monolayer on collagen could explain functional differences. Disaccharide analysis was performed using reverse phase HPLC with post-column derivatization and fluorescence detection. Expression of DSPG was analyzed by immunoblot and Western blot. Abundance of DS-specific 4 and 2 sulfotransferases was evaluated by real time PCR. Disaccharide analysis showed fibroblasts in 3D culture produced 11 fold more DS and chondroitin sulfate (CS) than fibroblasts in 2D culture. This analysis also revealed that all DS from 2D fibroblasts was unsulfated, while 89% of 3D fibroblast DS was 4-O sulfated. The DSPG decorin was abundant in extracts from 3D culture but absent in 2D fibroblasts while syndecans 1 and 4 remained unchanged. The abundance of DS-specific 4-O sulfotransferase transcript decreased by 85% in 3D culture, suggesting feedback inhibition. These data demonstrate that the change from 2D to 3D stimulates the synthesis of dermatan sulfate by fibroblasts. Given that the level of DS in 3D culture resembles the in vivo situation, and that 4-O sulfation of DS is required for activation of several growth factors, cytokines, and enzymes, this characteristic of the extracellular matrix of 3D fibroblast cultures may be essential to the function of such tissue constructs.

0257

New progress in fermentation technology: development of an elastin-stimulating yeast lysate

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It is well known that extracts for Saccharomyces cerevisiae (Baker's Yeast) that have been grown under conditions of cellular stress such as energy sources like heat or UV light, or application of toxic materials such as hydrogen peroxide causes the yeast to respond and produce defensive cellular components. These defensive components, called stress response elements, are typical of most eukaryotic cells and human skins cells are no exception. Because the stress response of yeast and human skin cells are so similar, stress elements isolated from yeast have found applications in skin care formulations. The characteristics of the yeast stress response can be influenced by the growth peptone that the yeast is grown in. This offers an opportunity to further influence the stress response through manipulation of the yesat growth media to stimulate the yeast to produce various cytokines and growth factors similar to human-based growth factors. We have begun to explore colorimetric methods for measuring key extracellular matrix proteins in skin cells and have developed an in vitro assay for measuring soluble elastin production in confluent human fibroblast cultures and in full thickness human tissue models. The method relies on a combination of technologies involving culturing of human testing substrate (cells or tissue), treatment of these substrates with the test materials and examination of soluble elastin production through a colorimetric assay that specifically isolates soluble elastin precursors. Using this testing methodology we have found that a new yeast lysate being developed in our laboratories appears to have a statistically significant positive influence on soluble elastin production in vitro.

0258

The integrin-matrix metalloproteinase-9 association and epidermal growth factor receptor/matrix metalloproteinase-9 signaling are regulated by gangliosides

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Gangliosides regulate cell migration on fibronectin(FN) by binding with the integrin α5 and inhibiting signaling through focal adhesion kinase(FAK). Although the predominant ganglioside of keratinocytes, GM3, affects cell migration, it does not bind α5 integrin to influence FAK signaling. Given the inhibition by GM3 of EGF receptor(EGFR) signaling and the regulatory role of EGF on expression of matrix metalloproteinase-9(MMP-9), a critical factor in cell locomotion, we hypothesized that gangliosides are able to modulate MMP-9 activity. Using antisense oligomers(to accumulate GM3), gene transfection of GD3 synthase(to deplete GM3), and gene transfection of ganglioside-specific sialidase and biochemical inhibition of ganglioside synthesis(to deplete gangliosides), we developed in vitro and mouse models of endogenous alterations of gangliosides. GM3 accumulation inhibited the migration on FN of the keratinocyte-derived SCC12 cell and dramatically decreased expression and activity of MMP-9. GM3 depletion facilitated both SCC12 cell migration on FN and cell invasive capacity. Mouse tumors with GM3 depletion grew 10-fold larger than controls. Both in vitro and in the mouse, GM3 depletion led to increased expression and activity of MMP-9, activation of both EGFR and MAP kinase, and increased AP-1 expression. Inhibitors of EGFR and MAPK phosphorylation blocked the expression and activity of MMP-9; these inhibitors of EGFR and MAPK, MMP-9 blocking antibody, and recombinant tissue inhibitor of metalloproteinase-1(TIMP-1) suppressed the increased migration induced by ganglioside depletion. Interestingly, endogenous accumulation of GM3 totally prevented the co-immunoprecipitation of MMP-9 and integrin $\alpha5\beta1$, whereas ganglioside depletion facilitated their co-immunoprecipitation.In conclusion, gangliosides regulate keratinocyte migration via both FAK and EGFR/MMP-9 signaling pathways,at least in part by modulating the ability of $\alpha5\beta1$ and MMP-9 to associate in the cell membrane.

0259

Production of anti-desmoglein3 monoclonal antibodies from pemphigus vulgaris mouse model produced by naive splenocytes adoptive transfer

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Pemphigus vulgaris (PV) is an autoimmune blistering disease caused by anti-desmoglein3 (Dsg3) IgG autoantibodies. Previously we have produced PV mouse model by adoptive transfer of splenocytes from Dsg3-/- mice immunized with recombinant Dsg3. Subsequently we isolated from the PV model mice several anti-Dsg3 monoclonal antibodies (AK mAbs), one of which was proved to be pathogenic. In this study, we attempted to produce PV model mice without active immunization and isolate monoclonal antibodies (NAK mAbs) from those mice. When 5x107 splenocytes isolated from naive Dsg3-/- mice were transferred about 80% of recipient mice developed the PV phenotype including oral erosions with suprabasilar acantholysis in histology. There were no apparent differences in the phenotype between the recipient mice with naive splenocytes and those with immunized splenocytes. The frequency of the phenotype appearance was dependent on the number of splenocytes transferred. We then isolated 8 NAK mAbs from the PV model mice produced by naive splenocytes. All of these clones reacted with mouse Dsg3 but not with mouse Dsg1 by ELISA and stained cell surfaces of cultured keratinocytes. When their hybridoma cells were inoculated in recipient mice, all of them showed in vivo IgG deposition in hard palates, but without apparent induction of the PV phenotype. The precise comparison of AK and NAK mAbs will provide valuable information to clarify the immunological mechanism of pathogenic antibody production against Dsg3 in PV model mice.

0260

Keratinocyte biointegration to poly (2 hydroxyethyl methacrylate) (pHEMA) implants

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Adverse complications from cutaneous insertion of medical devices such as catheters or glucose sensors, result in significant morbidity and mortality. Keratinocytes (KCs), stimulated to migrate, typically marsupialize along the sides of the implant, creating a space in which bacteria can invade and form biofilms. In order to preclude bacterial colonization or biofilm formation at this biomaterial/skin interface, the basal KC must attach or biointegrate with the device. To systematically study the biomaterial/skin interface, we developed a rafted organ culture (ROC) model using newborn foreskin implanted with porous poly (2-hydroxyethyl methacrylate) (pHEMA) rods. Since pHEMA, used for contact lenses, precludes protein adhesion, we chemically modified pHEMA with carbonyldiimidazole (CDI) and immobilized KC adhesion promoting proteins such as fibronectin (FN) and laminin 5 (Lam 5) to the surface of pHEMA. ROCs were grown for six days, fixed in 10% neutral buffered formalin (NBF) for paraffin embedding, fixed in 1/2 strength Karnovsky's for Epon embedding or frozen for embedding in O.C.T. then sectioned for morphology and immunohistochemistry. Our results show successful integration of migrating KCs into the pHEMA pores. The integrated KCs were characterized for general morphology, as well as for differentiation and proliferation markers by immunohistochemistry.

0261

Quantitative evaluation of molecules secreted by senescent human dermal fibroblasts cultured in monolayer versus collagen gel: description of an iterative treatment model

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At the moment, one of the main challenges in the field of dermatology is the understanding of cutaneous alterations which occur during the menopause. These changes are supplementary to other intrinsic (physiological) or extrinsic (photoaging) modifications. The hormonal deprivation of steroids and especially estrogens, accentuates some parameters of physiological ageing. The main signs of cutaneous ageing linked to the menopause are observed at the dermal level, as the reduction of water and collagen content results in a slimmer dermis. The acknowledged phenomenon of a decrease in the proliferative capacity of fibroblasts with ageing, may lead to a quantitative and qualitative reduction of the dermal macromolecules. Our objective was make the fibroblasts grow older through successive subcultures using in vitro methods and as time went on, we observed the changes in parameters implied in the ageing process : type I and III collagen, elastin, matrix metalloproteinase-1 and -2 (MMP), and interleukine 8. The behaviour of these in vitro aged fibroblasts was traced both in monolayer cultures and in three-dimensional cultures after inserting the cells into a collagen gel in order to mimic the cutaneous environment. In senescent fibroblasts, we observed a quantitative decrease in extracellular matrix protein synthesis associated with a quantitative increase in molecules implied in degradation : MMP-1 and IL-8. These phenomena were particularly noticeable in three-dimensional cultures. As our studies were orientated towards selecting ingredients able to modulate the parameters modified with ageing, we adapted our model to an iterative application over a period of 5 months. Our work was carried out using the soja plant as it has a high content of phytoestrogens, which are notorious for the treatment of mature skin.

0262

Novel role of angiotensin II in fibroblasts: induction of fibroblast migration by angiotensin II via HB-EGF-mediated EGF receptor transactivation

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Angiotensin II (AngII) has a well-defined role in controlling systemic blood pressure and volume homeostasis. Recently, AngII has been reported to accelerate wound healing in animal models. Therefore, we compared the wound-healing rate in AngII type 1a receptor (AT1aR)-deficient and wild-type mice. A cutaneous wound was made with an 8-mm punch biopsy and the wound area was measured on day 8. The wound area was 28.6 and 14.1 mm2 in AT1aR-deficient and wild-type mice, respectively. The AT1aR-deficient mice showed significant retardation of wound healing. This led us to examine the effect of AngII on fibroblast growth and migration using human fibroblasts. The presence of AT1R in human fibroblasts was confirmed by RT-PCR and a binding assay. AngII induced fibroblast migration in a dose-dependent manner, with a 1.9-fold optimum at 10-7 M, while AngII had no effect on fibroblast growth. Several groups have reported that ligand binding to the G-protein-coupled receptor (GPCR) induced EGF receptor (EGFR) transactivation via HB-EGF shedding (the proteolytic conversion of membrane-anchored HB-EGF to the soluble form). Since AT1R is a GPCR, we hypothesized that AngII-induced fibroblast migration is mediated by an EGFR transactivation mechanism. AngII induced the phosphorylation of EGFR, with a maximum at 30 min, and this was prevented by the presence of valsartant, an AT1R antagonist. Furthermore, valsartant also inhibited AngII-induced fibroblast migration by 90%. Anti-HB-EGF neutralizing antibody (3 mg/ml) inhibited AngII-induced fibroblast migration completely. CRM197, a non-toxic mutant of diphtheria toxin, specifically inhibits HB-EGF by binding to its EGF-like domain. CRM197 inhibited AngII-induced fibroblast migration almost completely at 1 mg/ml. In conclusion, we showed a new biological function of AngII in skin wound healing via enhanced fibroblast migration. Furthermore, this was mediated by EGFR transactivation via HB-EGF shedding through the AngII/AT1R signaling pathway.

0263

PUVA induces both cytoplasmic and nuclear changes that are responsible for a long-term growth arrest of human dermal fibroblasts

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We have shown previously that following single PUVA treatment human dermal fibroblasts undergo a long-term senescence-like growth arrest. However, the molecular mechanisms underlying this growth arrest are not known in detail. Nuclear effects as well as membrane damages have been attributed to be responsible. To address the question whether nuclear or membrane related damage seem responsible for the long-term growth arrest, we performed cell fusion experiments using karyoplasts of PUVA-treated fibroblasts and enucleated cytoplasts of normal fibroblasts as well as vice versa. Fibroblasts were stably transfected with GFP and neomycin as marker genes and treated with PUVA. At different time points post PUVA karyoplasts expressing GFP and neo(r) were fused with cytoplasts of parental fibroblasts labeled with latex beads. Hybrids were selected in G418 containing medium and the successfully fused cells showed GFP expression and included latex beads. Similarly, cytoplasts of PUVA-treated cells were stained with Hoechst-33342 before fusion to label contaminating nuclei after fusion and fused with karyoplasts of normal fibroblasts. Hybrids with cytoplasts or karyoplasts isolated from PUVA-treated fibroblasts up to one week post treatment died within 5 days. Hybrids with PUVA-treated cytoplasts and karyoplasts selected from time points at two to four weeks post treatment were viable and behaved in different way. Hybrids with PUVA karyoplasts from cells at 4 weeks after treatment proliferated for up to 17 days after fusion. In contrast, hybrids with cytoplasts from PUVA treated cells did not die but were temporarily growth arrested for up to 12 days after fusion. From these experiments we conclude that PUVA treatment induces damages in both the nuclear (DNA) and the cytoplasmic compartments. We hypothesize that cytoplast effects are less damaging compared to nuclear effects by PUVA. In addition, PUVA-induced factors in the cytoplast seem to have an ability to redirect the cells to a proliferating or growth arrest state.

0264

The Thy-1 molecule on activated human dermal microvascular endothelial cells specifically interacts with CD11b (αM-integrin) on leukocytes—impacts on inflammatory processes

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Previous work demonstrated that human Thy-1 is a newly identified activation associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). Thy-1 is involved in the adhesion of monocytes and polymorphnuclear cells (PNC) to activated HDMEC. In this study we identified the corresponding ligand of the Thy-1 molecule on these cells. First, we generated a monoclonal antibody (mAbAW2) against PNC and monocytes that blocks the binding of Thy-1 protein to its ligand. This antibody as well as the purified Thy-1 show identical binding patterns compared to an anti-CD11b antibody. Blocking experiments revealed that both, the mAb AW2 as well as the purified Thy-1 inhibit the binding of an anti-CD11b antibody to monocytes and PNC. These data suggest that Thy-1 binds to CD11b (αm-chain of integrins) on PNC and monocytes. Cell adhesion studies showed that mAbAW2 as well as anti-CD11b-antibodies were able to block the adhesion of PNC and monocytes to Thy-1 transfected CHO-cells as well as to Thy-1-expressing activated HDMEC emphasizing an essential role of Thy-1 / CD11b interaction for cell adhesion. The CD11b is a well characterized cell adhesion molecule on monocytes and PNC. Until now it has been thought that ICAM-1 is the main cellular ligand of CD11b. Our data first time provide evidence that human Thy-1 on activated HDMEC is a second cellular ligand of CD11b mediating the binding of monocytes and PNC to activated endothelium, possibly regulating invasion of leukocytes into inflamed tissues.

0265

Cutaneous microfibrils control transforming growth factor-β (TGF-β) activity through latent TGF-β binding protein-1

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TGF-β is a potent growth factor that enhances production of extracelluar matrix. TGF-β is involved in wound healing and fibrosis. Cutaneous microfibrils are mainly composed of fibrillin and an extracellular element with elasticity. Recently, latent transforming growth factor-β binding protein-1 (LTBP-1) has been found to interact with fibrillin and localize to microfibrils. Therefore, cutaneous microfibrils target TGF-β within dermal connective tissues via LTBP-1. However, mechanism how TGF-β is released from cutaneous microfibrils is unknown. Expression of both spliced and non-spliced forms of LTBP-1 by dermal fibroblasts was shown. Extraction studies using guanidine hydrochloride suggested non-covalent interaction between microfibrils and LTBP-1. Other extraction studies utilizing plasmin treatment revealed the presence of cross-linked LTBP-1 in aged skin. Immunohistochemical analysis after plasmin treatment also showed decreased staining of LTBP-1 in a concentration of plasmin dependent manner, suggesting that plasmin released LTBP-1 from cutaneous microfibrils. Western blot analysis of the treated solution showed consistent amount of release of LTBP-1 from dermal matrix. These results indicate that cutaneous microfibrils regulate TGF-β activity through proteolytic release of LTBP-1.

0266

The body-site specific expressions of the extra cellular matrix and integrin families explain the distinct features of the trunk, palm-sole and oral mucosa

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Epidermal keratinocytes are well known to change their biochemical and morphological characteristics depending on their body. For example, suprabasal keratin K1/K9 or K4/K13 are dominant in the palmo-plantar skin or oral mucosa, respectively, instead of the ubiquitous K1/K10 expressed in the trunk type epidermis. However, dermal components are not yet known for their distinct features among each body site. We thus used a combination of the subtractive mRNA screening and cDNA array to identify human cDNAs, which specifically and selectively expressed in the dermal fibroblasts established from each body site. In this process, two cDNA clones encoding a fibronectin and a pregnancy specific beta 1 glycoprotein are frequently identified in the fibroblasts derived from trunk skin, compared with the fibroblasts derived from other body sites. mRNA for fibronectin is highly abundant in the fibroblasts derived from trunk skins, compared with those of sole skins and oral mucosa. Whole skin extracts containing both epidermal and dermal components also revealed that sole skin and oral mucosa are apparently scarce for fibronectin whereas more abundant for vitronectin compared with trunk skin in the Western blot analysis. Meanwhile, various integrins were also distributed in the distinct spatially differentiation pattern. Trunk fibroblasts had more abundant integrins alpha 5 and alpha V than sole or oral mucous fibroblasts. Integrins alpha 4 and beta 1 showed no obvious difference among these fibroblasts. These tendencies of distinct expression are also confirmed in the immuno-histochemistry of various skin sites. Fibronectin is recently recognized to act as the migration guidance and the survival factors of neural crest cell, especially melanocytes. Our observations concerning about the paucity of fibronectin at palmo-planter skin and oral mucosa might well explain the reason why these body regions are less melanogenic regardless of the race and skin color types.

0267

Tetraspanins CD9, CD151, and CD81 are implied in the adhesion of normal human melanocytes

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Tetraspanins are a family of transmembrane proteins (25-50 kD) with at least 28 distinct members in mammals, that are abundantly expressed in different type of cells (endothelial cells, T and B lymphocytes, keratinocytes...). This family has been implicated in cell motility, adhesion, metastasis, cell proliferation, differentiation and intracellular signaling. Integrins are a large group of cell-surface ab heterodimers that function as key adhesion receptors for the extracellular matrix. Tetraspanins have been found to modulate signaling, compartmentalization, intracellular trafficking and recycling of integrins. Although some tetraspanins have been described in melanoma cells, their function have not been explored in normal human melanocytes. We found that normal human melanocytes express CD9, CD63, CD151, and CD81 by flow cytometry analysis. Fluorescence microscopy studies showed localization of CD9, CD151 and CD81 mainly at the cell membrane and in the intercellular junctions, with predominant intracellular expression of CD63. We studied their influence in the melanocyte adhesion and found that monoclonal antibodies against these molecules inhibit the adhesion to collagen matrix.

0268

Pemphigus vulgaris provides novel insights into keratinocyte differentiation

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We recently demonstrated that plakoglobin plays a central role in pemphigus vulgaris (PV). Here we further corroborate the involvement of this protein in the disease process. While keratinocytes from β-catenin knock-out mice responded to antibody binding with keratin retraction and loss of desmosome-mediated adhesion like wild type cells, plakoglobin knock-out cells did not. Moreover, mouse epidermal keratinocytes differentiated normally in the absence of β-catenin while plakoglobin knock-out cells formed a hyperproliferative epidermal sheet when grown at the air-liquid interface. Concomitantly, plakoglobin knock-out cells showed impaired upregulation of proteins such as desmoglein1 and loricrin which are characteristic for terminal differentiation. Collectively our results indicate that plakoglobin but not β-catenin is essential for differentiation of interfollicular epidermal keratinocytes. The fact that PV antibodies interfered with the localization of plakoglobin at the plasma membrane and in the nucleus therefore strongly suggests that the function plakoglobin exerts during normal keratinocyte differentiation is hampered in PV.

0269

Over-expression of retinoic acid receptorα in the cell-line COS-1 results in increased matrix metalloproteinase-1 production

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Retinoic acid receptor α (RARα) is increased in aged, photoprotected epidermis. In order to investigate the role of RARα in intrinsic aging, transient over-expression of this gene was performed in an *in vitro* model system, which was then examined for biomarkers of an aged phenotype. The gene encoding RARα was cloned into the plasmid pSG5 and transiently transfected into COS-1 cells. Whole cell lysates and media were harvested at 24 and 48 hrs post transfection and assayed for RARα and a panel of senescence-associated biomarkers: matrix metalloproteinase-1 (MMP-1), heat shock protein 70 (Hsp70) and p16 (n = 4). In additional experiments (n = 3), transfected cells were supplemented with 100nM all-*trans* retinoic acid (*t*RA; in dimethylsulfoxide) for 24 hours prior to sample preparation. Following transfection with 5mg of construct, consistent over-expression of the receptor was observed at both 24 and 48 hrs. Treatment of the cells with *t*-RA resulted in clearance of the receptor. MMP-1 was increased in the presence of over-expressed RARα at 48 hrs post transfection; the level returned to baseline following the addition of *t*RA. Constitutive levels of Hsp70 remained unaffected at both time points, and subsequent treatment with *t*RA had no effect. Likewise, p16 expression remained unaltered. This study identifies that over-expression of RARα results in increased production of MMP-1 and confirms that addition of *t*RA reduces these levels; this is in agreement with *in vivo* observations in aged skin following topical *t*RA treatment. Additionally, treatment with *t*RA directly reduces the artificially high receptor load in transfected COS-1 cells. It appears that RARα may play an important role in the mechanisms of intrinsic aging.

0270

Inhibition of matrix metalloproteinase activities by green tea polyphenols

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Green Tea Polyphenols (GTP) and its major component (-)-epigallocatechin gallate (EGCG) possess strong anti-oxidant, anti-inflammatory, and anti-carcinogenic properties. To substantiate the benefit of GTP and EGCG to matrix integrity, we have investigated their effects on several matrix metalloproteinase activities. Our target enzymes were MMP-1 (interstitial collagenase), MMP-2 (72 kDa gelatinase-A), MMP-3 (stromelysin-1), MMP-8 (neutrophil collagenase), and MMP-9 (92 kDa gelatinase-B). Activities were measured via digestion of fluorogenic peptide substrates, zymography using gelatin or casein substrates, and digestion of native substrate detected with SDS-PAGE. Flurogenic peptides were Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)-NH_2 for collagenase and gelatinase detection, and NBD-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(DMC)NH_2 for stromelysin detection. Using these substrates, 0.01% GTP and EGCG inhibited all of the targeted MMP activities by more than 90%. This efficacy was confirmed by gelatin zymography (MMP-2 and MMP-9), casein zymography (MMP-1 and MMP-3), and by digestion of purified human collagen type I with SDS-PAGE detection (MMP-8). The reversibility of inhibition was revealed by incubating MMP-2 with GTP and EGCG followed by gelatin zymography analysis. Our results suggested GTP and EGCG are capable of reversibly inhibiting the MMPs believed to be responsible for the major structural alterations of dermal matrix that are related to an aged appearance of the skin.

0271

Cysteine proteases initiate matrix degradation in early stages of melanoma invasion

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Assessment of the interactive mechanisms of matrix degradation during tumor invasion requires an *in vitro* model system closely reflecting the complexity of the *in vivo* situation. We established an optimized *in vitro* invasion system for melanoma cell lines using dead deepidermized dermis (DDD) as an appropriate model to analyze tumor cell migration, stroma degradation and changes in expression levels of matrix degrading proteases. Four melanoma cell lines (MV3, SkMel28, SkMel23, WM164) differing in their invasiveness were seeded either on the dermal side or on the basement membrane (BM) of the DDD. After two weeks of incubation all melanoma cells invaded the dermis, however, only MV3 and SkMel28 cells were also able to degrade the BM. To analyze the responsible protease activities, we performed *in situ* gelatin zymography of cryosections under two different conditions that employed neutral or acidic pH values to favor matrix metalloproteinases or cysteine proteinases (CPs), respectively. Gelatinolysis in this system generally correlated with the invasiveness of the cells. Under neutral conditions, gelatinolysis was detected in melanoma cells seeded on the dermal side but not when cultured on the BM. In contrast, at acidic pH, where CPs are preferentially active, proteolytic activity was detected in melanoma cells seeded on the dermis as well as in those seeded on the BM, suggesting that the activity of CPs is important for the early invasion process. Additionally, a modified method of gelatin zymography under special buffer conditions favoring the CPs revealed strong proteolytic activities in the supernatants of these cells, indicating the involvement of several CPs. The herein described tumor invasion system, combined with the new technique of acidic gelatin zymography, helps to improve our understanding of the complex mechanisms of matrix degradation and metastasis and revealed that CPs may crucially contribute to the invasion process.

0272

Disruption of β1 integrin induces keratinocyte stem cell anoikis through the extrinsic apoptotic pathway

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Cell survival requires interaction with the extracellular matrix and integrins control cell death in many cell settings. We have shown recently that keratinocyte stem cell (KSC) expressing highest levels of β1 integrin, are protected from apoptosis (anoikis). In this study, we analyze the molecular mechanisms underlyng anoikis of KSC caused by disruption of integrin signaling. KSC were isolated according to their adhesive capacity to type IV collagen and characterized by their expression of β1, α6β4 integrin, ΔN p63 as well as by the colony forming efficiency. TUNEL staining, PARP cleavage and flow cytometry analysis revealed that % apoptotic cells was significantly lower in KSC than in the more differentiated keratinocyte subpopulations (TUNEL, $p<0.05$; FACS, $p<0.01$). Anti-β1 integrin caused apoptosis in both KSC and more differentiated cells, while it decreased Bcl-2 and survivin protein levels in KSC. We next analyzed the caspase pathway during anoikis. Upon treatment with anti-β1 integrin, both initiator and effector caspases were activated earlier in more differentiated cells than in KSC. In particular, caspase-8 was activated at 3 hrs, bid at 3-6 hrs, caspase-3 at 3-6 hrs, while caspase-9 at 6-9 hrs. Inhibition of caspase-8 delayed the activation of caspase-9, caspase-3 and bid; moreover, inhibition of caspase-8 partially prevented KSC anoikis. On the contrary, inhibition of caspase-9 did not exert any effect on caspase-10, caspase-8 caspase-3 and bid, while it failed to protect KSC from anoikis. This study indicates that disruption of β1 integrin signal induces anoikis in KSC. The extrinsic apoptotic pathway seems to play a major role in this process.

0273

Keratinocyte stratification following ectopic desmoglein 1 expression *in vitro*; a possible mechanism for rising above the rest

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Desmoglein (Dsg) 1 is a member of the cadherin family of calcium-dependent cell adhesion molecules that is present in the desmosomes of the upper epidermis and is a molecular target in several human skin diseases. Previous studies have shown that normal human epidermal keratinocytes (NHEKs) fail to express Dsg1 but the expression of this desmosomal cadherin is markedly up-regulated in cells undergoing terminal differentiation. Using an organotypic model for epidermal differentiation, we confirmed that Dsg1 is restricted to the upper layers of differentiated cells that formed over time in these cultures. To define the role(s) of Dsg1 in keratinocyte differentiation, a retroviral gene delivery system was employed to force the expression of this cell adhesion molecule in undifferentiated NHEKs. The ability of ectopic Dsg1 to localize to cell borders and increase cell adhesion in infected NHEKs was directly related to the levels of extracellular calcium present in the culture medium. In spite of the largely cytoplasmic distribution of ectopic Dsg1 in NHEKs cultured at low calcium, the expression of this desmosomal cadherin was associated with keratinocyte stratification. Desmocollin 1, a desmosomal molecule expressed only at later stages of keratinocyte differentiation, was also up-regulated in these stratified cells. By incubating keratinocytes in a hanging drop of culture medium, we also observed the reorganization of NHEKs into multilayered cellular aggregates following Dsg1 expression in low calcium. In contrast, the ability of keratinocytes to stratify in culture was not altered by the overexpression of the related desmosomal cadherin subtype, Dsg3. Collectively, these studies suggest that Dsg1 expression is involved in promoting keratinocyte differentiation in a manner that is not directly related to its adhesive functions.

0274

Dermatan sulfate supports KGF-dependent keratinocyte proliferation and migration

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Glycosaminoglycans (GAGs) such as Dermatan Sulfate (DS) Heparan Sulfate (HS) and Hyaluronic acid (HA) are key regulatory molecules in development, maintenance and repair of the skin. DS is the predominant sulfated GAG in the dermis and is released in wound fluid after injury. Recently, we have shown DS is more potent than HS or other GAGs in its ability to act in vitro as a cofactor for FGF-7 (KGF). Unlike other members of the FGF family, KGF is a potent mitogen whose activity is a specific paracrine factor for cells of epithelial origin. To test the relevance of the activity observed for DS in the KGF model system, we investigated the ability of DS to promote cellular proliferation and migration of primary human keratinocytes. Cells in complete growth medium treated with exogenous DS repaired defects in their monolayer 2-3 days faster than untreated cells or cells treated with other GAGs. Keratinocytes in minimal medium showed increased proliferation in the presence of DS when they were treated with KGF. This effect was dose dependent and reversed at high DS concentrations (>50ug/ml) where DS inhibited growth. Evaluation of p44/p42 phosphorylation using KGFR-expressing cells deficient in endogenous GAG demonstrated that stimulation by DS was mediated through the rapid activation of MAPK by the KGF-DS-KGF-receptor complex. Finally, the ability and potency of DS to act as a cofactor for KGF was shown to be strictly dependent upon GAG structure. Minimal length and sulfation structure of DS required for KGF dependent signaling was defined as an iduronate-rich octasaccharaide containing extensive 4-O sulfation. These data demonstrate that DS released from the extracellular matrix acts as a cofactor for KGF-dependent signaling, and that release of this GAG is dose and sequence-specific. Interaction of carbohydrate sequence encoded in GAGs with amino acid sequence encoded proteins is an essential element of cutaneous biology.

0275

Role of zyxin, a focal adhesion phosphoprotein, in melanocyte migration and differentiation

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Focal adhesions link extracellular matrix proteins, integrins and the cytoskeleton as well as generate signals for cell spreading, proliferation and survival. We have shown that the expression of zyxin, a focal adhesion phosphoprotein involved in spatial control of actin assembly, is directly related to cell spreading and proliferation and inversely related to differentiation in melanoma cells and melanocytes. Here we investigate the relationship of melanocyte differentiation to cell migration and zyxin expression using isolated human neonatal melanocytes in serial passages, Western immunoblots and digital time-lapse and confocal microscopy. Early passage non- or hypo-pigmented diaphanous melanocytes exhibit high mean migration rates and numerous large zyxin-labeled focal adhesions whereas late passage stellate or dendritic pigmented melanocytes exhibit low mean migration rates and relatively few small zyxin-labeled focal adhesions. Actin bundle organization and complexity reflect zyxin localization and Western immunoblots confirm a decrease in zyxin expression as differentiation increases and migration rates fall. These findings support a role of zyxin in melanocyte migration and differentiation.

0276

The mouse desmosomal cadherin gene cluster contains a novel family member, desmoglein 4

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Desmogleins are members of the cadherin superfamily, and as such represent critical structural components of the desmosomal cell-cell junctions in keratinocytes and other epithelia. The genes encoding mouse desmogleins are localized within 350 kb of chromosome 18, within a desmosomal cadherin gene cluster also containing the three mouse desmocollin genes. Here, we describe a novel mouse desmoglein gene, designated desmoglein 4 (Dsg 4), which is localized within the desmosomal gene cluster, between Dsg1 and Dsg3. The gene arrangement of the desmosomal cadherin cluster in mouse is: Cen-Dsc3-Dsc2-Dsc1-Dsg1γ,Dsg1α,Dsg1β-Dsg4-Dsg3-Dsg2-Tel. Dsg4 has 40%, 40%, 40%, 34% and 43% amino acid identity to Dsg1α,β, γ, 2 and 3, respectively. Comparison of its exon-intron structure reveals a high level of evolutionary conservation expected from these related family members. In contrast to Dsg1α, Dsg1β and 3, whose expression is largely restricted to the skin, Dsg4 is also expressed in the brain, skeletal muscle, heart, kidney, among other tissues, suggesting a more widespread role in cell adhesion, in parallel with Dsg2 and Dsg1. Dsg4 is present in the human and rat genomes in the identical location, and a high degree of amino acid homology suggests functional importance of the gene and corresponding protein in mammalian skin. The identification of this novel desmosomal cadherin gene will facilitate mutation detection in mice with phenotypes manifesting in the skin and other tissues.

0277

A novel pemphigus vulgaris antigen, desmoglein 3-like molecule, is disrupted in desmoglein 3-/- mice that have pemphigus-like lesions

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Pemphigus vulgaris (PV) autoantibodies (Ab) to keratinocyte (KC) antigens (Ag) other than desmoglein (Dsg) 1 and 3 have been shown to induce PV-like lesions in Dsg3-/- mice (129xBL/6 Dsg3null strain). While PVAb absorbed by recombinant protein of extracellular (EC) domain of Dsg3 can induce PV-like lesion in neonatal mice, the Ab also strongly recognize other epidermal Ag of Dsg3-/- mice including a 130 kDa protein. I have hypothesized that the pathogenic Ab may target KC Ag with similar peptide sequence to Dsg3EC. Blast searching of the Genebank database for molecules with homology to Dsg3EC revealed a computational predicted novel Dsg3-like molecule. 3217bp reading frame of the molecule were amplified from human epidermal cDNA by overlapping RT-PCR. The cDNA encodes for a polypeptide of 117kDa that is 55% similar to Dsg3 and contains all 4 conserved regions of a cadherin. Since the EC of Dsg3-like molecule is more than 90% similar to Dsg3EC, I asked if Dsg3 PVAb could recognize the novel molecule. Recombinant protein of Dsg3-like EC was generated by pQE30 expression vector transformed into E. coli and used for Western blot. The protein appeared to be strongly recognized by affinity purified PVIgG to Dsg3EC. Unlike Dsg3-/- ancestors (B6; 129X1-Dsg3tm1Stan strain), only few rare older Dsg3-/- mice of 129xBL/6 Dsg3null strain were found to have lesions. Since Dsg3-like molecule contains an identical stretch of contiguous antigenic peptide contained in the immunogen previously used to generate antibody for testing the absence of Dsg3 in B6; 129X1-Dsg3tm1Stan mice, I suspected that these exceptional mice might inherit from their ancestors to be homozygous for disrupted Dsg3-like gene. 1278 bp cDNA coding for partial Dsg3-like homolog was amplified from mouse epidermal cDNA by RT-PCR. PCR genotyping showed 4 out of 5 exceptional mice to be homozygous for disrupted Dsg3-like gene in addition to disrupted Dsg3 gene. Thus the data suggest that novel Dsg3-like molecule may have an important role in mediating KC adhesion and it is one of the KCAg targeted by pathogenic PVAb.

0278

The triple-helical domain of type VII (anchoring fibril) collagen promotes human epidermal keratinocyte migration via $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins

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Recent studies suggest that anchoring fibrils and anchoring filaments are collapsed and in contact with the basal keratinocytes. Type VII collagen (COL VII), the major component of anchoring fibrils, consists of a central collagenous triple helical domain (TH) flanked by a 145 kDa N-terminal noncollagenous (NC1) globular domain and a small 34 kDa C-terminal noncollagenous (NC2) domain. In this study, we determined whether recombinant COL VII promoted the migration of human epidermal keratinocytes and dermal fibroblasts and identified the cell surface receptor involved. Recombinant COL VII vigorously promoted human fibroblast and keratinocyte migration. Cell migration on COL VII was divalent cation dependent and was inhibited by EDTA. Although COL VII contains four RGD sequences, the migration of keratinocytes and fibroblasts on COL VII was not inhibited by the presence of RGD peptides. By using function-blocking monoclonal antibodies, COL VII mediated cell migration was completely inhibited by monoclonal antibodies directed against the integrin subunits $\alpha 2$, $\alpha 3$ and $\beta 1$, but not by antibodies to $\alpha 5$, $\alpha 6$, and $\beta 4$. To further localize the domain within COL VII that promotes cell migration, we subjected a series of COL VII domain constructs, including the pure NC1 and NC2 domains and a truncated minicollagen that lacked 684-amino acids of central triple helical domain, to the migration assays. Using these purified mutant proteins, the migration promoting activity of COL VII was mapped to 684 amino acids (residues 1920-2603) within the triple helical domain. Further, two generated G2049E and R2063W mutations, next to the 39 amino acid helical interruption hinge region, that are associated with recessive dystrophic epidermolysis bullosa, showed significantly reduced ability to promote keratinocyte migration. We conclude that cell motility induced by COL VII is mediated by a 684 amino acid segment within the TH and by the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin receptors on the cell surface.

0279

Role of connective tissue growth factor in the pathogenesis of scarring in Steven Johnson Syndrome

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Stevens Johnson Syndrome (SJS) is a rare vesiculobullous disease characterized by an acute cutaneous eruption that involves the skin and mucosal membranes. Conjunctiva is one of the commonly affected mucosal surface in patients with SJS. In the advance stages, development of conjunctival fibrosis is associated with blindness in a significant number of patients with SJS. In the present study, we examined the role of transforming growth factor beta 1 (TGF-b1) and connective tissue growth factor (CTGF) in conjunctival scarring in patients with SJS. Biopsies from conjunctiva of 7 patients with SJS, and 5 control subjects, were studied for the expression of type I and type III collagens, TGF-b1, and CTGF, by using immunohistochemistry and quantitative real-time PCR. The role of TGF-b1 on the induction of collagen, and CTGF was also studied using fibroblasts isolated from conjunctival tissues. Compared to controls, an increased expression of interstitial collagens (type I and III) was detected in conjunctival biopsy sections of SJS patients, as detected by immunohistochemistry. Similarly, in contrast to the weak and sparse expression of TGF-b1 and CTGF in control conjunctiva, elevated expression of these fibrogenic molecules was observed in conjunctiva of patients with SJS. By quantitative real-time PCR, an increase in the expression of type I collagen (3.3 fold), TGF-b1 (1.7 fold) and CTGF (3.4 fold) was detected in the conjunctival tissues obtained from patients with SJS, compared to controls. When conjunctival fibroblasts were treated with TGFb1, upregulation in the expression of type I collagen, and CTGF was detected. Increased levels of TGF-b1 and CTGF might regulate increase synthesis and production of interstitial collagens, and thereby could contribute significantly to the development of conjunctival scarring in patients with SJS.

0280

Measurement of the mechanical properties of actin filament and keratin intermediate filament networks in a monolayer of cultured human epidermal keratinocytes

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Arguably, the biological basis of the robust mechanical properties of the epidermis can be contributed to (i) highly organized actin and keratin intermediate filament networks within individual keratinocytes, and (ii) the cell-cell junctions, namely desmosomes and adherens junctions, that anchor the cytoskeletal networks between adjacent keratinocytes into a functional tissue. Although many rheological studies have investigated the viscoelastic properties of *isolated* actin and keratin intermediate filaments, few researchers have attempted experiments geared towards quantifying the mechanical properties of *organized* and *anchored* cytoskeletal networks as they exist in a confluent monolayer of human keratinocytes cultured *in vitro*. Towards this end, an experimental blister-type, load-displacement apparatus has been designed and constructed to measure the dynamic differential pressures and deflections of a microfabricated, clamped, circular, elastic polydimethylsiloxane (PDMS) membrane upon which normal human keratinocytes have been plated and cultured into a confluent monolayer. Simple mechanical models of isotropic, bi-layered, linear elastic, incompressible membranes convert measured pressure-induced deflections of a composite substrate (PDMS + keratinocyte monolayer) into cellular stresses and strains. Therefore, pressure-deflection data collected from a composite substrate during a temporal study of adherens junction and desmosome assembly (induced by the well-known calcium switch) can be used to characterize the formation of organized and anchored cytoskeletal filament networks within the keratinocyte sheet. It is hoped that an advanced understanding of the constitutive mechanical behaviors of *organized* and *anchored* actin and intermediate filament networks may provide valuable insight into their respective biological functions within the epidermis in states of both health and disease.

0281

Increased expression of integrin-linked kinase correlates with melanoma invasion

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Cell adhesion to extracellular matrix (ECM) is an important process that controls cell survival, shape, migration, proliferation, and differentiation. Integrin-linked kinase (ILK), a key component of the cellular ECM adhesion, has been studied extensively in recent years. Overexpression of ILK in epithelial cells results in anchorage-independent cell growth with increased cell cycle progression. Furthermore, increased ILK expression is correlated with progression of several human tumor types, including breast, prostate, brain, and colon carcinomas. To investigate the role of ILK expression in melanoma progression, we employed the tissue microarray technique and examined the ILK expression in 67 human primary malignant melanoma biopsies by immunohistochemistry. Our data showed that ILK was highly overexpressed in 16 of 67 cases (24%). We found that overexpression of ILK was significantly correlated with the thickness of the tumors ($P<0.05$). Of the six cases which have lymph node metastases, five showed overexpression of ILK in primary tumors. Furthermore, our analysis also indicated that melanoma patients older than 60 years of age had more ILK overexpression than those younger than 60 years ($P<0.01$). Overexpression of ILK was not associated with the histological types or the location of the tumors. Taken together, our data suggest that ILK overexpression may play an important role in melanoma progression.

0282

Characterization of molecular mechanisms underlying mutations in dystrophic epidermolysis bullosa using site-directed mutagenesis

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Type VII collagen (COL VII) is the major component of anchoring fibrils, structures which mediate epidermal-dermal adherence. COL VII gene (COL7A1) mutations cause dystrophic epidermolysis bullosa (DEB), a genetic mechano-bullous disease, but the underlying molecular mechanisms leading to DEB clinical phenotypes are unknown. In this study, we introduced 4 individual substitution mutations, G2049E, R2063W, G2569R and G2575R, into the COL VII expression vector using site-directed mutagenesis. All four mutations result in a total absence of anchoring fibrils in recessive DEB patients. The mutant constructs were transfected into human 293 cells and stably transfected cell clones isolated. All but R2063W synthesized and secreted a 290 kDa mutant COL VII alpha chain at levels similar to the wild type COL7A1. Biochemical characterization of the purified mutant COL VII proteins and structural and functional comparisons with wild type COL VII demonstrated: (1) The G2569R and G2575R mutations near the end of the triple helical domain adjacent to noncollagenous NC2 resulted in mutant COL VII with an increased sensitivity to protease and a decreased ability to form trimers. (2) The G2049E and R2063W mutations next to the 39 amino acid helical interruption hinge region produced mutant COL VII with reduced ability to support keratinocyte and fibroblast migration. (3) Limited proteolytic digestion of mutant G2049E and R2063W proteins yielded aberrant fragments and revealed reduced stability of the triple helix. These mutations cause a local destabilization of the triple-helix that exposes an additional highly sensitive proteolytic site that maps within the region of the mutation. The R2063W mutation also resulted in the secretion of an unstable degraded 200 kDa COL VII mutant protein. We conclude that these COL7A1 mutations are located within motifs of different importance for the function of COL VII. Protein processing, folding, stability, and cell-matrix binding may be altered by specific mutations in the COL7A1 gene.

0283

Essential oil of neroli is a potent inhibitor of MMPs activities

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Induction and activation of matrix metalloproteinases (MMPs) including MMP1, MMP2, MMP3 and MMP9 has been demonstrated to play a crucial role in skin photoaging. The aim of this study was to identify inhibitors of MMP1, MMP2, MMP3, MMP9 activities and synthesis in order to counteract the degradation of the extracellular matrix components. To this purpose, several plant extracts were evaluated for their inhibitory potential. Extracts were incubated with MMP activated with p-aminophenyl mercuric acetate. The enzymatic activity was monitored by the addition of a fluorescent substrate specific for the respective MMP. We observed an inhibition of the activities of the 4 aforementioned MMPs with the essential oil of Neroli (EON) (Bigaradier,*Citrus aurantium*, extract) and an extract of dried Green Tea leaves (GTL) (*Camellia Sinaesis*) rich in flavanols which was used as a positive control. Inhibition of MMPs activity, up to 100%, was dose dependent and the MMP2 inhibition was confirmed by gelatin zymography. No regulation of MMP1, pro-MMP2, pro-MMP9 expression by keratinocytes was detected at the concentrations used. Our data demonstrate the essential oils may contain potent inhibitors of MMPs and therefore represent interesting candidates for the treatment of skin disorders due to MMP activation.

0284

Targeted inhibition of human collagenase-3 (MMP-13) expression by antisense ribozyme inhibits squamous cell carcinoma growth *in vivo*

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Squamous cell carcinomas (SCCs) of the head and neck are characterized by high tendency to invade locally and metastasize to lymph nodes. Collagenase-3 (MMP-13) is specifically expressed by tumor cells of SCCs. To specifically examine the role of MMP-13 in the growth and invasion of SCC, we constructed a hammerhead ribozyme targeted against human MMP-13 mRNA. The anti-MMP-13 ribozyme effectively cleaved MMP-13 transcripts in vitro. Delivery of MMP-13 antisense ribozyme into metastatic cutaneous SCC cells with a recombinant adenovirus, RAdMMP-13ASRz, resulted in potent and specific inhibition in production of proMMP-13 and suppressed invasion of SCC cells through Matrigel by 80%. In vivo injection of RAdMMP-13ASRz into human SCC xenografts established in SCID mice inhibited tumor growth by 50-75%, inhibited MMP-13 expression and gelatinolytic activity, and reduced number of proliferating cells by 30%. The MMP-13 antisense ribozyme delivery also resulted in apoptosis in SCC cells within 72 hours. These results show, that MMP-13 plays an important role in SCC tumor growth in vivo identifying MMP-13 as a potential therapeutic target in SCCs of the head and neck.

0285

Keratinocytes grow at the medium/air interface on a basement membrane-like sheet formed by cell-released proteins

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Epithelial cells require adherence to a matrix for regular growth. During standard keratinocyte cell culture in serum-free medium, we observed that cell colonies formed not only on the bottom of the culture vessels but also at the medium/air interface. Coomassie blue staining detected a protein membrane that extended up to several centimeters between the colonies of floating cells. Ultrastructural investigation of this membrane revealed structures that closely resembled those of basement membranes and immunochemical staining confirmed the presence of laminins-1 and -5, representative components of basement membranes. Cells attached to the floating sheet were proliferating and could be cultivated for up to six months. Our finding that matrix proteins released by keratinocytes are able to self-assemble into basement membrane-like sheets gives new insight into the formation of the epithelial/mesenchymal interface. The fact that these membranes can sustain continued cell growth opens new ways for cultivation of epithelial cells.

0286

Regulation of ADAM9 expression in human skin tumors

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ADAM9 belongs to a family of metalloprotease disintegrins (ADAMs) which are membrane-anchored glycoproteins thought to be involved in protein ectodomain shedding and cell-cell or cell-matrix interactions. However, the precise biological functions of ADAM9 are still unclear. The aim of this study was to analyze the regulation of ADAM9 expression in tumor cells in vitro and in vivo in human skin tumors. RNA analyss of melanoma cell lines with different invasive abilities showed ADAM9 expression in all cell lines independent of their invasive capacities. ADAM9 expression did not depend on cell density, was not altered by growth factors and cytokines, and by culture on different extracellular matrix proteins (i.e. fibronectin, type IV collagen, monomeric collagen type I) used as planar substrates. However, we observed down-regulation of ADAM9 expression upon culture of melanoma cells within three-dimensional lattices of fibrillar type I collagen, whereas, culture within three-dimensional gels consisting of alginate, a polysaccharide, did not alter transcript levels. These results indicate a specific regulatory effect of fibrillar collagen on ADAM9 expression. In various skin tumors, by immunohistochemical stainings, we found an intense staining of ADAM9 primarily in basal keratinocytes and in some suprabasal cells. In contrast, basal cell carcinoma cells displayed in most cases no or a faint staining. In malignant melanoma, ADAM9 appeared to be more diffusely distributed and the staining restricted to the melanoma cells within the invading part. Surprisingly, ADAM9 protein was not only detected in the melanoma cells but also in peritumoral stromal fibroblasts while it lacked in fibroblasts distant of the tumor site. These findings suggest that in vitro ADAM9 expression is poorly regulated by cell proliferation and soluble factors while fibrillar collagen has a putative novel regulatory role on ADAM`s expression. In addition, the distinct distribution pattern of ADAM9 in human skin tumors implicates a potential role of this protease in the aberrant phenotype displayed in neoplasia.

0287

Human melanoma cells induce synthesis of proMMP1 in co-cultured dermal fibroblasts by secreting bFGF and IL-1α

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Proteolytic degradation of the extracellular matrix is one major event in invasion and metastasis of malignant melanoma. This process is achieved by the concerted action of different proteolytic enzymes including the matrix metalloproteinases (MMPs). Using in situ zymography we have shown that in human melanoma increased MMP activity is localized at the border of tumor cells and the peritumoral connective tissue emphasizing the role of tumor-stroma interactions in the modulation of the enzyme activity. To study the mechanisms of tumor-stroma interactions we have co-cultured BLM melanoma cells and human dermal fibroblasts (dFb) either in direct cell-cell contact or in transwell chambers, in which the cells, separated, can communicate by soluble factors. In both systems synthesis of proMMP1 was increased. Culturing dFb in the presence of media from BLM cells resulted in a strong induction of proMMP1 synthesis suggesting that soluble factors are responsible for this induction e.g. IL-1αand bFGF that are known to be potent inducers of proMMP1 synthesis and are produced by the BLM cells. Culture of dFb with IL-1αand bFGF were indeed effective in inducing MMP1 synthesis. Competition of IL-1αwith recombinant IL-1RA and neutralizing antibodies, dose-dependently decreased but failed to completely abolish synthesis of proMMP1. Similarily, bFGF antibodies dose-dependently inhibited proMMP1 synthesis suggesting that this induction is caused by multiple soluble factors. In human melanoma tissue we observed increased IL-1αand bFGF protein in the melanoma cells corroborating the role of these factors in vivo. We also showed that in vitro melanoma cells produce increased amounts of IL-1αupon contact with connective tissue proteins e.g. type I collagen. In conclusion, these results suggest that the contact of melanoma cells with dermal matrices might induce synthesis and secretion of soluble factors, eg. IL-1αthat in turn can activate dermal Fb to produce proteases.

0288

Dsg1δ is a novel member of the mouse desmosomal cadherin cluster

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The mouse desmogleins are members of the desmosomal cadherin superfamily, and are key structural components of the desmosome. Like in the human genome, the genes encoding mouse desmogleins are tightly clustered within 350 kb of chromosome 18, within a desmosomal cadherin gene family also containing the three desmocollin genes. We have characterized a novel mouse desmoglein gene, which is highly homologous to both mouse and human Dsg1, and have designated it desmoglein 1γ (Dsg1c). Dsg1γ shares 83% amino acid identity to the previously described mouse Dsg1, now designated as Dsg1α, and 32% and 40% identity to mouse Dsg2 and 3 respectively. The Dsg1γ gene maps within the desmosomal gene cluster, between Dsc1 and Dsg1α. Comparison of its exon-intron structure reveals a high level of evolutionary conservation with related family members. In contrast to Dsg1α and Dsg3 whose expression is largely restricted to the skin, Dsg1γ is also expressed in the brain, skeletal muscle, and liver, among other tissues, and is thus more similar to Dsg2 in its tissue distribution. Thus, the gene arrangement of the desmosomal cadherin cluster in mouse is: Cen-Dsc3-Dsc2-Dsc1-Dsg1γ,Dsg1α,Dsg1β-Dsg4-Dsg3-Dsg2-Tel. Interestingly, an orthologous Dsg1δ gene was not found in the current version of the human or rat genomes, suggesting that the desmosomal cadherin gene cluster contracted during mammalian evolution.

0289

Venous leg ulcers and apoptosis: a TIMP-3 mediated pathway ?

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Tissue inhibitor of metalloproteinase-3 (TIMP-3) belongs to a family of secreted proteins that regulate the activity of several metalloproteinases and is an inducer of apoptotic cell death. The prodeath domain of TIMP-3 resides in its N-terminal region and apoptosis is induced by the activation of a signaling cascade involving caspase-8 and -9. Venous leg ulcers account for the majority of chronic wounds and lack clinical signs of necrotic events. Here we determined the protein expression of TIMP-3, FAS/CD95 by immunohistochemistry, the caspase-8 and caspase-9 protein expression and activity by caspase activity assays as well as the DNA-fragmentation by positive DNA in situ nick end-labeling (TUNEL) in tissue specimens of venous leg ulcers or healthy skin controls. Staining with anti-TIMP-3 displayed an intense signal around collagen bundles in the reticular dermis of venous leg ulcers. Staining with Fas/CD95 showed an intense cellular immunoreactivity in lesions in comparison with healthy controls. Immunoblotting with caspase-8 and -9 antibodies revealed prominent bands for the processed forms as well as the active subunits of these apoptosis regulators. Lesional skin displayed strongly increased activity for caspase-8 and caspase-9 in comparison with healthy controls measured by caspase activity assays. Moreover, quantification of apoptotic cells by TUNEL revealed significantly elevated apoptotic cell death in venous leg ulcers in comparison with healthy controls. In summary our data for the first time provide evidence that venous leg ulcers could be the result of an apoptotic signaling pathway, which might be induced through a TIMP-3-mediated inhibition of proteinase-dependent ECM-degradation. These results suggest that controlling programmed cell death may have therapeutic potential in preventing venous leg ulceration.

0290

A novel human desmosomal cadherin gene family member, desmoglein 4

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The human desmogleins are members of the desmosomal cadherin superfamily of proteins, and are critical structural components of the desmosome. Desmogleins serve as autoantibodies in acquired bullous dermatoses such as pemphigus vulgaris and pemphigus foliaceous. In addition, inherited mutations in desmoglein 3 result in the bal/bal phenotype in mice, and human desmoglein 1 mutations result in a form of striated keratoderma. Clearly, desmogleins are important determinants of cell-cell adhesion between keratinocytes of the skin and hair follicle. The genes encoding human desmogleins are localized within 350 kb of chromosome 18q12, within a desmosomal cadherin gene cluster also containing the three desmocollin genes. In this study, we characterized a novel human desmoglein gene, designated desmoglein 4 (DSG4), and mapped it within the desmosomal gene cluster, between DSG1 and DSG3. DSG4 has 38%, 36% and 46% amino acid identity to DSG1, 2 and 3, respectively. Comparison of its exon-intron structure reveals a high level of evolutionary conservation expected from these related family members. In contrast to DSG1 and 3, whose expression is largely restricted to the skin, DSG4 is also expressed in the brain, skeletal muscle, heart, kidney, pancreas and thymus, among other tissues. DSG4 orthologs with high homology are also present in the mouse and rat genomes in the identical location between Dsg1 and Dsg3, suggesting functional importance of the gene and corresponding protein. Interestingly, the newly identified Dsg1 isoforms in the mouse genome, Dsg1α and Dsg1β, are not represented in the human genome. Currently, the gene arrangement of the desmosomal cadherin cluster in the human genome is: Cen-Dsc3-Dsc2-Dsc1-Dsg1-Dsg4-Dsg3-Dsg2-Tel. The identification of this novel desmosomal cadherin gene will facilitate mutation detection in human disorders with cell adhesion defects in the skin and other tissues.

0291

The 97 kDa and 120 kDa fragments of type XVII collagen have different N-termini

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Type XVII collagen is a hemidesmosomal transmembrane glycoprotein. Several subepidermal blistering diseases are associated with an autoimmune response to this molecule, including linear IgA disease (LAD). Two proteolytic fragments of type XVII collagen are the major antigenic targets in LAD, including epidermis-derived 97 kDa (LABD97) and keratinocyte-derived 120 kDa (LAD-1) polypeptides. Both fragments were assumed to have the same N-terminus within the membrane-proximal 16th non-collagenous A (NC16A) domain of type XVII collagen. However, in contrast to LABD97, the N-terminus of LAD-1 has not yet been identified. To address this issue, in a first set of experiments, we immunoadsorbed serum from a rabbit, immunized against the recombinant NC16A domain, using 9 overlapping fragments covering this entire domain [amino acid (aa) 490 to 565]. Antibodies purified against a peptide covering aa 521-532 still labeled LAD-1 indicating that its N-terminus is different from that of LABD97 (aa 531, alanine). To determine the exact cleavage site of LAD-1, we performed N-terminal sequencing of the protein affinity-purified from concentrated medium of cultured keratinocytes (DJM-1 cells). This revealed leucine at aa position 524 as the N-terminus of the polypeptide. Interestingly, this position was 7 aa upstream of the N-terminus of LABD97. Mass spectrometry of protease-digested fragments of LAD-1 demonstrated the presence of fragments containing aa 528, confirming that the N-terminus of LAD-1 is different from that of LABD97. These data suggest that the 120 kDa and 97 kDa fragments are generated through different proteolytic processes from type XVII collagen.

0292

Mice deficient in neonatal Fc receptor (FcRn) are resistant to pemphigus foliaceus

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Pemphigus foliaceus (PF) is an IgG autoantibody-mediated cutaneous blistering disease. PF autoantibodies bind to the extracellular domain of desmoglein-1 (Dsg1), an epidermal cell adhesion molecule, and lead to a progressive epidermal cell-cell detachment known as acantholysis. PF autoantibodies are pathogenic as demonstrated by passive transfer into neonatal mice. The serum titers of PF autoantibodies correlate with disease activity and extent of the skin involvement. The FcRn mediates the intestinal absorption of maternal IgG in neonatal rodents and the transplacental transport of maternal IgG in humans. Recent studies have suggested that FcRn also plays a role in protecting circulating IgG from catabolism. The aim of this study was to test a hypothesis that mice deficient in FcRn are protected from PF because lack of the FcRn, an IgG protective receptor, results in accelerated degradation of pathogenic autoantibodies. To test this hypothesis, wild-type and FcRn-deficient mice were injected intraperitoneally with Dsg1-affinity purified pathogenic autoantibodies from PF patients and examined clinically, histologically, and immunologically for PF disease phenotypes. We found that FcRn-deficient mice were resistant to experimental PF. Circulating levels of PF autoantibodies in FcRn-deficient mice were significantly reduced compared to normal mice. These results indicate that FcRn regulates the serum IgG levels by protecting circulating IgG from degradation and blocking FcRn function could control antibody-mediated autoimmune diseases such as PF.

0293

Cloning and characterization of kindlin, a novel actin cytoskeleton and focal adhesion-associated protein expressed in keratinocytes

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Numerous cytoskeletal and cell junction proteins are known to have critical roles in keratinocyte migration and adhesion, although it is likely that further, as yet unidentified proteins might also contribute. We report a new structural protein, kindlin, that we recently identified as being abnormally expressed in the autosomal recessive skin fragility-poikiloderma disorder, Kindler syndrome (OMIM 173650). The gene for this protein, designated *KIND1*, is located on 20p12 and encodes a 677 amino acid peptide with homology to the focal adhesion protein, talin. Like talin, kindlin has a FERM (band4.1/ezrin/radixin/moesin) domain suggesting a role in linking the actin cytoskeleton to the plasma membrane. This novel protein also has a pleckstrin homology domain and may therefore function in signal transduction. Multiple tissue northen blotting of keratinocyte cDNA revealed strong expression of a 4.9kb transcript in colon, kidney and placenta and lower expression levels in heart, skeletal muscle, liver and small intestine. An additional 5.8kb transcript was also present in colon. There was an absence of expression in spleen, lung and peripheral blood leukocytes. Further northern blotting and RT-PCR showed the transcript was expressed in cultured keratinocytes but not in cultured fibroblasts. A kindlin-GFP fusion protein was shown to co-localize with the actin cytoskeleton and also with vinculin, a component of focal contacts, in both keratinocytes and PtK2 cells. In summary, we have identified a new keratinocyte structural/signaling protein that is associated with the actin cytoskeleton and focal contacts and which is abnormally expressed in Kindler syndrome.

0294

Analysis of the dermo-epidermal junction in mice lacking the basement membrane proteins nidogen-1 and nidogen-2

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Nidogens are a small family of related proteins which includes the two mammalian species, nidogen-1 and nidogen-2. Based on biochemical and structural studies it has been proposed that nidogens act as connecting elements for basement membrane (BM) assembly. To elucidate this role further, mice deficient in either nidogen-1 or both isoforms were generated. Nidogen-2 deficient mice for cross-breeding had been kindly provided by B. Skarnes (University of California, Berkely, USA) and characterized by B. Bader (Max-Plank-Institute for Biochemistry, Martinsried, Germany). The absence of either nidogen-1 or nidogen-2 did not affect BM formation in general. By contrast, mice lacking both nidogen isoforms die shortly after birth showing a wide range of developmental defects. Surprisingly, the gross morphology of skin appeared normal with no signs of blister formation. In addition, no differences in epidermal keratinization between mutant and wild-type skin were observed. So far immunostaining with other BM components did not reveal major differences at the dermo-epidermal junction in mutant skin when compared to wild-type controls. However, at the ultrastructural level disruption and thinning of basement membranes as well as aberration in hemidesmosomes were detected, being currently analyzed in greater detail. Furthermore, we investigated BM synthesis and assembly in three-dimensional cocultures using human epidermal keratinocytes or HaCaT cells and skin fibroblasts from newborn nidogen-deficient mice. With NID1 -/-//NID2 +/- fibroblasts neither a distinct deposition of nidogen-1 or nidogen-2 was detectable in contrast to control cultures with wild-type fibroblasts. Surprisingly, there was also no deposition of laminin-10, but of some collagen IV, while laminin-5 was only slightly affected. Integrin alpha6beta4 appeared largely normal.

0295

Interaction of Bia-1 with integrin α1 tail regulates collagen expression

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Interaction of fibroblasts with extracellular matrix proteins influences a large number of cellular functions. The contact with most ECM proteins is mediated by integrin receptors of the β1 family, and binding of the integrin cytoplasmic domains to intracellular proteins is thought to initiate signal transduction. A yeast-two hybrid screen was performed, resulting in identification of proteins which bind to the conserved membrane-proximal region and others which bind either to several or exclusively to individual integrin cytodomains. Of the specific binding partners for α1 integrin, one was further analyzed. Termed Bia-1, it represents an as yet unknown protein which displays the structural organization characteristic of the TRIM protein family: RING finger, zinc-binding and coiled-coil domains and a B30.2 box, which is indicative of protein interactions and potential dimerization. To study the biological function of Bia-1 and its possible role in modulating collagen synthesis, specific antibodies were produced for immunostaining and immunoprecipitation. Further, fibroblasts were engineered to either overexpress Bia-1 or expression was reduced by transfecting antisense oligodeoxynucleotides. In both approaches, Bia-1 and collagen (α1 or α2 (I)) mRNA levels were regulated in opposite directions, i.e. low Bia-1 levels correlated with high collagen synthesis. These results were confirmed by culturing untransfected primary skin fibroblasts under conditions of well-characterized modulation of collagen synthesis. These results strongly support a role for the cytoplasmic protein Bia-1 in initiating the signaling cascade elicited by activation of α1β1 integrin and regulating collagen synthesis.

0296

Emerging phenotypes of mice lacking integrin α2β1

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Cell - collagen interactions play vital roles in development and tissue homeostasis. Integrin α2β1 is one of the most important and abundantly expressed receptors which mediates these interactions. To study α2β1 functions in vivo, we generated mice lacking the α2 subunit. Surprisingly, mice deficient in α2β1 develop apparently normally and reproduce, indicating that no major developmental defects arise from α2-deficiency. The skin of these animals is normal at gross morphological inspection. However, functional analysis of fibroblasts and keratinocytes cultured from the skin of α2-/- animals revealed impairment in attachment, migration, contractile capacity, protease secretion as well as cytoskeletal abnormalities, all required for normal wound healing. Further, α2β1 was postulated as the major collagen receptor on platelets, mediating platelet adhesion to collagen and required for the formation of a hemostatic plug following injury. Indeed, differences in adhesion and collagen-induced aggregation by α2-deficient platelets were observed. However, bleeding times and platelet counts were normal in α2-/- mice. We then applied antibodies to block another collagen receptor, GPVI, which completely abrogated adhesion to collagen fibrils. Furthermore, intravital microscopy following endothelial injury demonstrated normal thrombus formation in vivo in α2-deficient mice. These results confirm that integrin α2β1 plays a supportive rather than essential role in hemostasis.

0297

Unidirectional organization of alpha-smooth muscle actin microfilament network is lost in aged human dermal myofibroblasts

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Skin myofibroblasts (MF) participate in wound healing contraction and dermal neoformation. Their contractile activity is attributed to a well-developed cytoplasmic microfilament network composed of myosin and actin, particularly alpha-smooth muscle actin (alpha-SMA), expressed throughout the philogenetic tree in cells whose main function is contraction. MF are connected to the extracellular matrix by specialized structures called fibronexi, where actin microfilaments and extracellular fibrils are organized into a close association, mediated by the integrin family of adhesion receptors. alpha-SMA expression was investigated by direct immunostaining on 8 normal dermal fibroblast (DF) strains from abdominal skin of healthy females (24 to 60 year old) after 10 days treatment with 1 ng/ml TGF-beta1, a potent inducer of the MF phenotype. Indirect immunostaining of integrin-beta1 and cell adhesion measurement on coated type I collagen were also performed. Alpha-SMA expression was stimulated by TGF-beta1 in all strains (by a factor 2 to 20) but no significant differences were observed between young and old strains the latter exhibiting the largest heterogeneity. Interestingly, confocal microscopy showed a multidirectional alpha-SMA microfilament network in older cells, the younger ones showing an unidirectional feature. Adhesion was greater for MF than for DF according to the higher integrin-beta1 expression measured on these cells but no statistical differences were observed between the two age groups. We hypothesize that the impairment of wound strength as a function of age may be due to a dispersion of the contractile forces throughout the multidirectional actin microfilament network of MF, more than a reduced ability of aged cells to express alpha-SMA in response to cytokines or a defect in their adhesion properties.

0298

Cystein-rich protein (CYR61), a mediator of collagen homeostasis, is elevated in chronologically aged, photoaged, and UV-irradiated human skin *in vivo*

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Chronologically aged, photoaged, and UV irradiated human skin have in common reduced synthesis of procollagen and elevated expression of matrix-degrading metalloproteinases (MMPs). Molecular mechanisms responsible for these abnormalities in chronologically aged, photoaged, and UV irradiated human skin remain largely unknown. Cystein-rich protein (CYR61) is an extracellular matrix-associated protein, which has been implicated in regulation of collagen and MMP expression. We have investigated expression of CYR61 in chronologically aged, photoaged, and UV irradiated human skin *in vivo*, and its functions in human skin fibroblasts. Laser capture microdissection coupled real-time RT-PCR and immunohistology revealed that CYR61 mRNA and protein were predominantly (80%) expressed in dermal cells in human skin. Over-expression of CYR61 in human skin fibroblasts, by adenoviral vectors, reduced type I procollagen mRNA expression (60%, n=3) and increased MMP-1 (2-fold, n=3), MMP-3 (2-fold), and MMP-9 (3-fold) mRNA expression. CYR61 mRNA levels (measured by quantitative real-time RT-PCR) were increased 3.9 fold in sun-protected aged (80+years) human skin, compared to young human skin (20-30 years, n=6, $p<0.05$). CYR61 mRNA levels were increased 2.9 fold in photoaged forearm skin, compared to subject-matched sun-protected hip skin (n=4, $p<0.05$). UV-irradiation (2MED solar-simulated UV) of human skin *in vivo* transiently induced CYR61 mRNA with maximum induction of 6.6-fold at 8 hours post UV (n=5, $p<0.05$). CYR61 gene expression returned to its initial basal level 24 hours post UV. These data demonstrate the ability of CYR61 to regulate MMP and collagen expression in human skin fibroblasts. Elevated expression of CYR61 may be a key mediator of aberrant collagen homeostasis observed in aged and photoaged human skin. Suppression of CYR61 expression represents a novel therapeutic target to improve the health of aged and photoaged human skin.

0299

Molecular mechanisms of connective tissue remodeling induced by CO_2 laser resurfacing of human skin *in vivo*

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Relatively little is known regarding molecular mechanisms of dermal extracellular matrix remodeling following CO_2 laser resurfacing for photodamaged skin. We have investigated expression patterns of key genes involved in dermal matrix remodeling following CO_2 laser treatment. Focal areas of photodamaged forearms were treated with two passes using the Coherent Ultrapulse laser (300mJ, 60W). Treatment resulted in complete removal of interfollicular epidermis. Total RNA was extracted from full thickness skin samples, and mRNA levels of specific genes were quantified by real-time reverse transcriptase PCR. Specific proteins were localized by immunohistochemistry, using frozen sections. CO_2 laser resurfacing resulted in rapid and marked induction of primary cytokines IL-1β and TNF-α (maximal induction of 14-fold and 11-fold, respectively within 3 days). IL-1β and TNF-α are known to induce matrix-degrading metalloproteinases (MMP) and repress type I and type III procollagen (COL) expression. Maximal induction of MMP-1 (collagenase, 39,130-fold); MMP-3 (stromelysin, 1,040-fold), and MMP-9 (gelatinase B, 61-fold, n=10) was observed within 6 days post treatment. MMP-1 and MMP-3 mRNA levels returned to near baseline by 28 days post treatment, whereas MMP-9 remained elevated. TGF-β is the major regulator of procollagen production. TGF-β1 and TGF-β3, but not TGF-β2, were induced within seven days, following the peak of MMP expression, and remained elevated for 28 days. Increased expression of type I and type III COL coincided with increased TGF-β expression. COL I and III mRNA levels continued to rise for at least 28 days (maximally increased 6-fold and 5-fold, respectively). Increased expression of tropoelastin, fibrillin-1 and fibrillin-2 mRNA was observed at 28 days post treatment. CO_2 laser resurfacing biochemically affects repair of photodamaged skin by initially inducing MMP-mediated removal of actinically damaged collagen, and subsequently stimulating deposition of newly synthesized collagen.

0300

The armadillo family protein p120 modulates cadherin expression by regulating cadherin internalization and degradation

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The cellular mechanisms that regulate cadherin levels are poorly understood. Cadherins are coupled to the actin cytoskeleton by the armadillo family protein β-catenin, which binds to the c-terminal domain of classical cadherins. p120 is also an armadillo protein, but p120 binds to the cadherin juxtamembrane domain. In the present study, we sought to determine the mechanisms by which armadillo family proteins regulate cadherin expression in microvascular endothelial cells (MEC). To address this question, a mutant cadherin comprising the extracellular domain of the IL-2 receptor and the VE-cadherin (VE-cad) cytoplasmic tail was expressed in MEC. The mutant cadherin caused the rapid internalization and degradation of endogenous VE-cad, an effect that was mediated by the endosomal-lysosomal pathway. Interestingly, removal of the β-catenin binding domain from the mutant cadherin tail had no effect, but mutation of the p120 binding domain ablated the ability of the mutant to trigger endocytosis of endogenous VE-cad. Over-expression of p120 but not beta-catenin increased endogenous VE-cad levels and rescued the down-regulation of VE-cad caused by the mutant cadherins. Furthermore, VE-cad levels were dramatically reduced in MEC in which p120 levels were knocked down using siRNA. To determine the mechanisms by which p120 regulates cadherin expression, MEC were treated with chloroquine to inhibit lysosomal degradation and thereby trap intracellular pools of VE-cad that entered the endocytic pathway. Over-expression of p120 inhibited the accumulation of intracellular VE-cad in chloroquine treated cells, whereas β-catenin had no effect. These data indicate that p120 modulates cadherin expression in MEC by regulating cadherin internalization and lysosomal degradation. These findings suggest a new paradigm in which the cellular levels of p120 function as a set point mechanism that controls cellular levels of cadherins.

0301

Putative role for protein kinase D in adherens junction formation

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In order to elucidate the role of the anti-differentiative protein kinase D (PKD) in keratinocyte maturation, we studied PKD's effect on adherens junction (AJ) formation in cultured mouse keratinocytes (KCs). Using the calcium switch to promote AJ formation, we treated KCs with various PKD/protein kinase C (PKC) inhibitors to determine their effect on AJ formation. We monitored AJ formation by isolating the TritonX-100 insoluble fraction from KCs, and then western blotted this fraction for E-cadherin, an AJ protein. We observed that compounds that potently inhibited PKD and PKC induced AJ formation, while compounds that inhibited PKC, but did not inhibit PKD effectively, had little effect. Thus we hypothesize that PKD's anti-differentiative role in keratinocyte biology is through, at least in part, inhibition of AJ formation.

0302

Human papillomavirus can be present in malignant melanoma but does not worsen the prognosis

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Although the aetiology of malignant melanoma is still unknown, environmental, hormonal and genetic factors have been implicated in its pathogenesis. These include lower skin phototypes, with fair or red hair and blue eyes, the amount of sun exposure and previous sunburns, a familial predisposition and increased number of dysplastic nevi. The role of human papillomavirus (HPV) in many neoplastic diseases, including cervical cancer and non-melanoma skin tumors, has been widely studied. Occasionally, HPV has been detected in some biopsy specimens from malignant melanoma, suggesting that HPV can be correlated with a more rapid melanoma progression and a poorer clinical outcome. In this study, to further investigate the presence of HPV DNA, we performed two different PCR-ELISA methods on 51 malignant melanoma, in comparison with 33 dysplastic nevomelanocytic nevi (Clark's nevi) and 15 healthy skin. We utilized two different consensus primer sets to optimize the detection of HPV DNA and, in particular, the consensus primer pair MY09 and MY11 to amplify a 450 bp segment of the L1 gene of 30 HPV genotypes and the consensus primer pair GP5+ and GP6+ to amplify a 150 bp segment of the L1 gene of 24 HPV genotypes. In both experiments, amplified products were separately hybridized with type specific biotynilated probes for high-risk HPV genotypes (HPV-16, 18, 31, 33, 35, 39, 45 and 52). HPV DNA was detected in 7 (14%) melanoma and 5 (15%) dysplastic nevi with MY PCR-ELISA, while with GP PCR-ELISA, HPV was identyfied in 11 (21%) melanoma and 8 (24%) dysplastic nevi. All HPV positive samples in MY PCR were positive also in GP PCR. The presence of HPV in malignant melanoma was not correlated to Breslow thickness microstage or other severity parameters. Our results suggest that HPV can be present in melanoma and in other melanocytic conditions, however we did not find a direct association between the virus and the melanoma progression.

0303

Human intracisternal A-type particle retrovirus proteins in systemic sclerosis patients

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Systemic sclerosis (SSc) is a connective tissue disease considered to be an autoimmune disorder. Although both cellular microchimerism and molecular mimicry of some common infectious agents, such as cytomegalovirus and parvovirus B19, have been implicated in its pathogenesis, the aetiology of SSc remain unknown. Human endogenous retroviruses (HERVs) have been identified in Sjoögren sindrome, insulin-dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus (SLE) and congenital heart block. Serum antibodies specific for human intracisternal A-type particles (HIAP), a human endogenous retrovirus recognized by monoclonal antibody against HIV-1 p24 capsid protein, have been associated to primary biliary cirrhosis, SLE, familial erosive arthritis and some patient with SSc. In this study, we performed western blot studies on 42 SSc patient sera, in comparison with 18 sera from primary biliary cirrhosis (PBC) patients and 52 healthy subjects (blood donors) to investigate a possible reactivity to HIAP retroviral proteins. Western blot reactivity to two or more HIAP proteins was detected in 10 (55.5%) serum samples from PBC patients and in 28 (66%) SSc patients. Only one healthy subject (1.9%) showed positive results to HIAP proteins. Our results showed a strong association between HIAP seroreactivity and the detection of autoantibodies to ds-DNA and antimitochondrial, anticentromere and anti-Scl70 antibodies. HIAP reactivity may be attributable either to an autoimmune response to antigenically related cellular proteins or to a specific antibody process, as consequence of a pathological process. Although the direct involvement of an A-type endogenous retrovirus in the aetiology of autoimmunity is still an open question, we can consider that endogenous retrovirus, HIAP proteins in particular, are an hallmark of autoimmune disease.

0304

Three-dimensional structures of cutaneous-free nerve endings during epidermal regeneration

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The aim of the study was to investigate re-innervation of cutaneous nerves in granulation tissue of burn wounds. Newly epithelialized skin tisuues just attached to the granular tissues of burned ulcer were obtained during debridement procedure. Obtained skin specimen were fixed with 0.01% glutaraldehyde and 0.1% paraformaldehyde in 0.1M phosphate buffer solution and they were immersed in 0.2% EDTA-2Na/ 125 Unit/mL dispase to facilitate separation of the epidermis from the dermis. The epidermal components were carefully removed using microforceps under the stereoscopic microscope. The separated dermal surface was observed three-dimensionally by the scanning electron microscope (SEM) and subepidermal nerve network was studied by the immunofluorescence antibody method (IF) with protein gene product 9.5. Distribution of substance P (SP) and calcitonin gene-related peptide (CGRP) and expression of the nerve growth factor receptor(NGFR) were also examined by the double-labeled IF of PGP 9.5 and SP, CGRP or NGFR and were viewed under the confocal laser scanning microscope. Results: SEM showed that numerous short length axon-like structures with a diameter and length of about 1 and 10 micrometer respectively, were seen penetrating through the basal lamina and one of these structures ended as a terminal expansion. Cell body of a terminal Schwann cell appeared on the basal lamina and possessed long slender dendrites. It was observed that there was an increase of PGP 9.5-positive fiber networks, arranged parallel and vertical against the granular tissues. The CGRP-positive substances appeared as granules on the PGP 9.5-positive fibers, but the SP-positive substances were seldom seen. Expression of the NGFR were also seen prominent on the elongated PGP 9.5-positive fibers. These results confirm that dermal re-innervation has a strong relationship with epidermal regeneration.

0305

Characterization of ST2L expression in circulating lymphocytes from patients with atopic dermatitis: up-regulation of ST2L in atopic dermatitis

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Th2/Tc2/NK2 cells have been suggested to express ST2L in vivo. We examined ST2L expression in peripheral blood lymphocytes from patients with atopic dermatitis (AD), which has been postulated to be a Th2-mediated disease, and then analyzed the possible correlation between these values and the levels of several clinical parameters. ST2L expression in peripheral blood CD4+, CD8+ T cells and CD56+ NK cells from 23 AD patients and 11 sex- and age-matched healthy control subjects were studied by flow cytometry. The percentages of ST2L+ cells in CD4+, CD8+, CD56+; cells were significantly higher than those in healthy controls subjects (ST2L+ in CD4+: 13.5±6.2% vs 4.7±2.7%, $p<0.0001$, ST2L+ in CD8+: 9.6±5.4% vs 3.1±1.1%, $p=0.0140$, ST2L+ in CD56+: 10.8±5.7% vs 2.2±0.7%, $p=0.0001$). The percentage of ST2L+ cells in CD4+CD45RO+ T cells correlated positively with the total serum IgE, LDH, eosinophil counts and skin scores in AD patients. The frequency of ST2L+ cells in peripheral blood lymphocytes might be a useful maker of disease activity in AD.

0306

CD56: a useful marker for diagnosing Merkel cell carcinoma

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Merkel cell carcinoma (MCC) of the skin is an aggressive but rare malignant neuroendocrine tumor. For its pathological diagnosis, we use a panel of immunohistochemical markers, such as cytokeratin 20 (CK 20), epithelial membrane antigen (EMA), chromogranin A, neuron specific enolase (NSE), synaptophysin, and Leu7 (CD57) to demonstrate its epithelial and neuroendocrine features. CD56, or neural cell adhesion molecule (NCAM), has been demonstrated recently as the tumor marker of the pulmonary neuroendocrine cell system. Its expression in MCC, however, has still rarely been investigated. Furtheremore, in such very few previous studies on NCAM expression in MCC, all the tumor cells were not necessarily demonstrated to express NCAM. Therefore, to study the immunoreactivity of CD56 in MCC, especially using a monoclonal antibody of a clone 1B6, different from those adopted in the previous reports, we reexamined CD56 expression immunohistochemically in 5 MCC cases, along with the conventional panel of markers described above, using paraffin-embedded tissue sections. CD56 revealed the most diffuse and intense positive staining, which was noted along the cell borders, in all specimens compared with other neuroendocrine tumor markers. The results of our study indicate that CD56, especially a new monoclonal antibody (clone 1B6), is a useful immunohistochemical marker for MCC.

0307

Stereological analysis of substance P expression in patients with delusions of parasitosis

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Delusions of parasitosis (DOP) is the unshakable conviction that one is infested by parasites, despite contradictory findings from an appropriate clinical evaluation. Our objective was to investigate the possibility of a neurogenic component being responsible for the abnormal sensations that these individuals experience. We hypothesized that the expression of the neuropeptide substance P (SP) and/or calcitonin gene-related peptide (CGRP) is altered in individuals with DOP. Four-mm punch biopsy specimens were taken from affected and non-affected skin of two patients diagnosed with DOP. Control skin biopsy specimens were site, age, and sex matched. Biopsy sections were triple labeled with primary antibodies to protein gene related peptide (PGP 9.5), SP, and CGRP, and then secondary antibodies conjugated to either Cyanine-3.18 or -5.18. All sections were subsequently labeled with Ulex europaeus agglutinin (UEA I) conjugated to fluorescein. Image data sets were collected with a BioRad MRC1024 confocal imaging system. Nerve and blood vessel densities were quantified using stereology with a special emphasis on epidermal sensory fibers. Neuronal SP expression was increased in affected and non-affected skin of individuals with DOP compared to controls. The density of SP-positive sensory fibers was 2-3 fold greater in affected vs. non-affected skin. PGP, CGRP, and UEA 1 expression were variable. Increased SP expression in affected skin of individuals with DOP suggests that this neuropeptide may play an important role in the altered sensory perception these patients experience.

0308

Methods for the evaluation of health economic studies in dermatology

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The warranty of adequate dermatological treatment provided by public health insurances is restricted by low budgets. Economic studies should analyse the utilisation of available resources. Four main categories, all dealing with incidental expenses, basically differ between their method of evaluation. The techniques calculate on anticipated costs and consequences. Scientific assessment mainly results from cost-minimisation (CMA), cost effectiveness (CEA), cost utility (CUA) or cost-benefit analysis (CBA). CEA is an adequate technique for expected varying results of comparable treatments measured in natural units (i.e. life-years gained, years of healed leg-ulcers) and not appropriate for the comparison of different medical sectors and to decide about allocation resources. CMA is a special form of CEA used in cases of alternative medications or treatments turn out to have the same result to identify the one with the lowest cost. The proof of effectiveness is a pre-requisite. Similar to CEA is CUA. There is a defined outcome (changes in the patients well-being) which is measured in QALYs (Quality adjusted life-year). If the consequences of an intervention are not identical, CBA is used and the benefit measured in monetary units. The assessment of the performed economic evaluations is aggravated by the usually low economical knowledge of physicians. A check-list of 30 criteria was created including six crucial points to differentiate the significance of health economic studies. The main concern concentrates on the justification for interventions/alternatives, the choice and naming of the kind of economic evaluation, sources of estimation for efficiency/(primary) outcome and separately listed prices and currencies. Discounting was performed, sensitivity and incremental analysis were undertaken. Fulfilled crucial criteria were rated with three points, the other criteria got one point. Good clinical studies achieved 40-48 points. This checklist is a good tool for clinicians to assess health economic studies.

0309

Withdrawn.

0310

Psoriasis vulgaris and Malassezia yeasts: an immunological study

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The role of Malassezia (M) species in psoriasis is, as yet undetermined, but there are several reports indicating that these yeasts have been implicated as a trigger for the initiation of scalp lesions. The purpose of our study was to evaluate the relationship between different Malassezia species isolated from scalp and cellular immune-status of forty psoriatic patients. Samples were obtained by scraping the skin surface on the scalp lesions and inoculated on modified Dixon agar plates. The yeasts were identified following the scheme established by Guillot et all. Additionally, we performed two-color flow cytometry analyses to investigate the lympohocyte subpopulations. The most common isolated species was M.globosa, found in more than a half of all isolates, followed by M.slooffiae, M.restricta, M.furfur and M.sympodialis. We demonstrated a significant increase in the percentage of suppressor T cells and a significant decrease in the percentage of helper/suppressor T cell ratio as well as NK cells. However, we find no correlation between isolated species and the changes in the peripheral blood lymphocyte subpopulations.

0311

The kinetic changes of confocal histopathology of allergic and irritant contact dermatitis *in vivo*

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Reflectance-mode confocal microscopy (RCM) is a novel technique that enables non-invasive imaging in vivo. It has previously been used to describe the histopathological features of contact dermatitis. This work was designed to investigate the kinetic changes of both allergic and irritant contact dermatitis (ACD and ICD) using this non-invasive technique to better understand their pathogenesis. Subject volunteers with a diagnosis of ACD were patch tested with the allergen and sodium lauryl sufate (SLS) and the results were compared against appropriate controls. RCM and other non-invasive methods including transepidermal water loss (TEWL) and fluorescence excitation spectroscopy (FES) were performed at 3 or more time points within up to three weeks after patch removal. Statistical analysis of RCM results and FES was performed by MANOVA and 2D-ANOVA respectively. Wilcoxon test for paired data was used to analyse TEWL results. Whereas parakeratosis, stratum corneum disruption and the presence of individual corneocytes are early events in ICD, similar changes can occur later in ACD. Cutaneous disruption induced by irritants rapidly returns to normal whereas the changes in ACD can last up to four weeks. RCM demonstrates significant differences in the increase of epidermal thickness between ACD and ICD ($p < 0.01$, days 2 and 3) and between ICD and control ($p< 0.05$ for all timepoints). Similarly, the difference in TEWL between ACD and ICD, as well as between ICD and control was significant at all time points ($p < 0.020$). FES on the other hand, while it showed significant differences between ACD and ICD ($p< 0.02$) did not show significant differences between ACD and controls. We conclude that non invasive optical methods such as RCM, TEWL and FES offer significant advantages to study the quantitative parameters of kinetic changes in acute contact dermatitis reactions.

0312

Morphologic spectrum of halo nevi including dysplastic changes: a study of 103 cases

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Halo nevi are benign proliferations of melanocytes with a characteristic clinical appearance. The histopathologic features include a dense lymphocytic infiltrate admixed with benign appearing melanocytes that contain varying amounts of melanin. Although halo changes have been described in commmon and Spitz's nevi, there is little information in the literature regarding the morphologic spectrum of the melanocytic component. We reviewed a large series of halo nevi paying attention to the presence of dysplastic changes. We studied 103 cases diagnosed as halo nevus between 1994-2000 for dysplastic changes by light microscopic examination of architectural pattern, degree of cytologic atypia, inflammation, and stromal changes. We assessed the degree of peripheral depigmentation in all cases by Fontana-Masson stain. In 12 cases with very dense lymphocytic infiltrates, we evaluated the expression of melanocyte-associated antigens: S-100 protein, Melan-A, bcl-2 protein, HMB-45, and c-kit. Sixty-three out of 103 halo nevi had features of dysplastic nevi. In 8/103 cases, we identified evidence of regressed dysplastic nevi such as lamellar fibroplasia and bridging of rete ridges. Thirty-six out of 103 were common nevi: 15 of these showed melanocytic enlargement and crowding of nests of melanocytes within the dermis and four showed complete regression of melanocytes. Four out of 103 were Spitz's nevi. In 12/12 densely inflamed halo nevi, melanocytes expressed S-100 protein, Melan-A, bcl-2 protein, and HMB-45, but in 11/12 cases c-kit staining was weak to absent. In contrast, the only densely inflamed halo nevus which we stained that strongly expressed c-kit in junctional and dermal melanocytic nests showed dysplastic features. These data expand the morphologic spectrum that can be seen in the melanocytic component of halo nevi. In our series of 103 halo nevi, we identified features of dysplastic nevi in 61%. We also report that 11/12 densely inflamed halo nevi had decreased melanocytic expression of c-kit; however one case with dysplastic features strongly expressed c-kit.

0313

The involvement of apoptosis-related proteins in primary and metastatic lesions of human malignant melanomas

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In the previous study, we have found that the decreased rate of TUNEL positive melanoma cells was significantly correlated with the poor prognosis of melanoma patients, where the rates of Fas and FasL positive cells were also lowered. For a further understanding of the regulation of melanoma cell apoptosis and its relationship to prognosis, we investigated the specimens from primary and metastatic lesions of the melanoma patients, which were divided into stage 3 (n=7) and 4 (n=7) (AJCC staging system for cutaneous melanoma, 2002), by TUNEL staining and immunohistochemistry for Fas, FasL, Bcl-2 and Bax. As a result, there was no significant difference in TUNEL positive rates between primary and metastatic lesions. The correlation between TUNEL- and Fas-, or FasL-positive rates in melanomas was significant in primary lesions and metastatic lymph nodes. Also, there were significant correlations in primary lesions between the positive rates of TUNEL and Bcl-2, or Bax in melanoma cells. Moreover, in the lymph nodes, the rate of TUNEL-, Fas- and FasL-positive melanoma cells, as well as the ratio of Bax positive rate to Bcl-2 positive rate (B/B ratio) of melanoma cells in stage 4 melanomas was less than that of stage 3 melanomas. Among these positive rates of melanoma cells in primary lesions and lymphocytes in both primary lesions and lymph nodes, there was no difference between stage 3 and 4. Therefore, it is possible that the apoptosis of melanoma cells and infiltrating lymphocytes in primary and metastatic lesions may be regulated by the Fas/FasL and the Bcl-2/Bax systems, and that the expression of the Fas system and the B/B ratio of melanoma cells in metastatic lymph nodes may affect the prognosis of the patients through the regulation of apoptosis induction, while the apoptosis of infiltrating lymphocytes may not influence the prognosis of the patients.

0314

Immunomodulator effect on erythema of rosacea

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This study was a 12-week open label clinical trial in which patients were treated with 0.1% Tacrolimus Topical Ointment (TTO)for an off lable indication, rosacea. Twenty-four patients with rosacea identified as having predominantly either erythrotelangiectatic (ET) or papulopustular (PP) subtypes were enrolled (13 ET and 11 PP). Erythema was statistically improved in both groups. Most subjects disliked the consistency of the ointment base used in TTO. Five patients withdrew from the study; most of these reported an increase of papulopustular lesions. We hypothesize that the observed benefit is due to down regulation of local immune and inflammatory processes. These findings suggest that the role tacrolimus on the vascular system in subjects with rosacea.

0315

CD30/CD7 ratio in lymphomatoid papulosis

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The diagnosis of many cutaneous malignancies can be problematic, and usually requires the use of immunohistochemical studies. CD7 is often used when diagnosing mycosis fungoides (MF), as deletion of this marker has been shown to be both sensitive and specific for diagnosis of mycosis fungoides. Lymphomatoid papulosis (LyP) is a CD30+ lymphoproliferative disorder that has long been associated with hematologic malignancies, especially mycosis fungoides (MF), but the degree to which CD7 is expressed in LyP has not been studied. This study was designed to: 1) determine the relationship between CD30 and CD7 expression in LyP as compared to MF; 2) evaluate the CD30/CD7 relationship in specimens read as "dermatitis" from the same group of patients; and 3) compare CD7 deletion between patients with isolated LyP and patients with both LyP and MF. All available tissue blocks from patients (N=19) with LyP were stained both CD30 and CD7 and assessed using a semiquantitative 0-4 scale. Concurrent diagnoses from these patients included MF (n=6) and dermatitis (n=13); these specimens were also studied. A total of 58 tissue samples were evaluated from 19 patients. This included 32 LyP, 6 MF and 21 dermatitis specimens. The mean+/-SD CD30 expression was 2.39+/-2.04, 0.67+/-1.03 and 1.00+/-0.95 for LyP, MF and dermatitis respectively (p=0.026). The mean+/-SD CD7 expression was 3.10 +/-1.22, 0.83+/-0.98 and 2.33+/-1.68 for LyP, MF and dermatitis respectively (p=0.010). The results showed that there was a statistically significant decrease in CD7 expression, without CD30 upregulation, in both MF and dermatitis lesions from patients with the diagnosis of LyP. They also confirm the increase of CD30 expression in LyP and the loss of CD7 expression in MF reported previously. Our results suggest that the CD30/CD7 ratio may be a useful tool in evaluating suspicious eruptions presenting in the context of LyP.

0316

Oral dexamethasone tablets for pulse therapy in pemphigus

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High-dose glucocorticoid pulse therapy aims at periodically strong immunosuppression and may reduce the daily maintenance dose of glucocorticoids, thus limiting the hazards of continuous long-term steroid intake. Pulse therapy is usually given intravenously rather than orally, without evidence to support the necessity of the intravenous route. Oral therapy is preferable because it avoids venipunctures, decreases costs, and improves patient convenience. In this study we investigated the bioavailibity and tolerability of 300 mg oral dexamethasone in pemphigus patients. Four patients with pemphigus vulgaris were enrolled in this study. Tablets containing dexamethasone 50 mg were produced in our hospital pharmacy. The dexamethasone European Pharmacopoeia (Eur Pharm) was hydrophylized with methylcellulose 15 MPA-s (Eur Pharm). The tablets were prepared by direct compression using cellulose mycrocrystalline (Eur Pharm, Avicel pH 101) and magnesium stearate (Eur Pharm). Serum samples were drawn after intravenous and oral pulse administration and analyzed by a validated, suitable, selective HPLC procedure. The area-under-the curve (AUC) of oral dexamethasone was 55.8% (range 43-65%) of that of intravenous dexamethasone. Mean peak concentration (Cmax) after intravenous administration of dexamethasone 200 was 5040 µg/L; the mean Cmax after an oral dose was 2580 µg/L, with mean time to Cmax of 2.25 hours. Oral high dose dexamethasone was well tolerated. With a bioavailibity of 55,8% oral administration of dexamethasone 358 mg is equivalent to an intravenous dose of dexamethasone 200 mg. Since it is unknown whether the effects of pulse therapy are due to AUC or Cmax, we concluded that oral dexamethasone 300 mg in 50-mg tablets can be used as oral pulse therapy. The new dexamethasone tablets have reliable pharmacologic and technical characteristics and appear to be safe. They are suitable for use in clinical trials such as PEMPULS (www.pempuls.nl), in which the therapeutic effect of oral high-dose dexamethasone pulse therapy is evaluated.

0317

Clinical evaluation of topical sodium L-ascorbyl-2-phosphate lotion (10%W/V) in patients with acne

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The etiology of acne vulgaris involves a complex relation among several causes or factors in the pathogenesis of the disease. Ascorbic acid (vitamin C) is contained in many kinds of cosmetics and there are many reports on its physiological effects, such as the inhibition of melanogenesis and prevention of lipid peroxidation. Moreover its strong anti-oxidization effects and inhibitory effects of sebaceous secretion have been reported. L-ascorbyl-2-phosphate (abbreviated as APS) (which is produced by SHOWA DENKO K.K.) is dissolved in water, so we expect that the solution containing APS may be effective for acne vulgaris. Here for the first time, we report on the clinical evaluation of 10% APS lotion used by patients with acne vulgaris. Eighteen subjects with acne (18 women), 18 to 22 years of age were enrolled in this study. This was an open comparison study. These patients were instructed to apply the 10%(W/V) APSL topically to the acne lesions on one side of the face only, twice a day, after washing the face in the morning and the evening. Treatment was continued for 8 weeks. At the beginning and at 4 weeks and 8 weeks of treatment, patients were observed for clinical response. The clinical response of each patient was evaluated by an observation of both sides of the face and rated on a 5-point scale. Clinical improvements of inflammation, and papules-pustules were obtained in 83.3% and 94.4% of the APSL treated side, but only 5.6% and 16.7% in the untreated side of the faces respectively. This is the first time the evaluation of topical 10%APSL in patients with acne has been reported.

0318

Hormonal effects on psoriasis in pregnancy

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Hormonal changes in estrogen and progesterone are believed to result in altered immune surveillance of autoimmune diseases such as psoriasis. Psoriasis tends to improve during pregnancy, possibly due to a hormone-induced down-regulation of the immune system. All prior research on psoriasis during pregnancy has been retrospective, often with the patient completing the survey several years after pregnancy. This is the first study which quantifies how psoriasis fluctuates in pregnancy and postpartum and measures women's hormone levels (estradiol, estrone, estriol, progesterone) in pregnancy to determine which, if any, correlate with psoriatic change 1200 patients were screened at UCI and Long Beach Memorial prenatal offices. 85 patients were enrolled (55 study group, 30 control). Data collection July 2000 - April 2003. Severity of psoriasis assessed and blood drawn at 10, 20, 30 wks of pregnancy and 6 & 24 wks postpartum. * Improved in pregnancy 52% (no change 24%, worsened 24%) * Worsened postpartum 62% (no change 31%, improved 7%) * Out of women with >15% body coverage who improved, the psoriatic plaques present 1st trimester cleared 85% by 3rd trimester * Progesterone did not correlate with psoriatic change; estrogen did correlate with psoriatic improvement * Ratio of estrogen-progesterone had the most significant correlation with improvement (r-squared of 0.62) Estrogen, and not progesterone, correlated with psoriatic improvement. Estrogen is a member of the nuclear receptor family, which includes retinoids, vitamin D, and thyroid derivatives that are currently used to treat psoriasis. We believe that this research has the potential to be useful in developing new clinical approaches to the management of psoriasis as well other autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and linear IgA disease, which have fluctuation patterns similar to psoriasis in pregnancy.

0319

Phenotype does not affect anthralin-induced inflammation

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There is considerable inter-individual variation in response to cutaneous stimuli, but what factors underpin this variability and what proportion can be explained by genetics? We wished to determine what role hair and skin pigmentation plays in the inflammatory response induced by anthralin, including effects of covariables on anthralin-mediated tolerance. 83 volunteers of whom 26 were red heads, range 16-72 yrs (median 38yrs) and skin types I-VI, had doubling doses of anthralin in Lassars paste (LP) from 0.005-0.08% applied in duplicate to the back for 24 hours using 8mm Finn chambers (Epitest). In addition 30 volunteers -12 red heads- had a 4cm x 4cm patch of mid back skin pretreated with 0.005% and 0.01% anthralin in LP, under occlusion, for 24 hours on successive days. A similar size patch of LP was applied to the opposite side of the back as a control. On Day 3 each site was challenged for 24 hours with LP, 0.02, 0.04 and 0.08% of anthralin in LP using 8mm Finn chambers. The inflammatory response was quantitatively assessed on Day 5 using the Moor laser Doppler. This was validated for skin pigmentation by measuring temperature dependant change in flux in 10 individuals of skin types I-VI. Hair samples were assayed for melanin content using HPLC. Hair colour was classified using the L'Oreal hair colour standards chart in addition to Tristimulus colourimetery (Minolta Chroma Meter) to objectively measure hair colour and skin colour from photo exposed and photo protected sites. As expected there was significant inter-individual variation in anthralin dose response curves ($P<0.01$) and more than a 60% reduction in erythema at the induced dithranol sites ($P<0.01$). When comparing skin types I-III and IV-VI there was a significant % difference in flux between the control and induced site at 0.04% anthralin ($P<0.01$), but otherwise neither hair colour, skin colour nor skin type had any effect on anthralin induced inflammation or tolerance ($P>0.1$). Thus in contrast to UV radiation, pigmentary phenotype cannot be used to predict anthralin mediated inflammation or tolerance.

0320

Kinetics of response to therapy in pemphigus vulgaris

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The response of PV to therapy is usually expressed in terms of partial or complete remissions, endpoints that may take several years to reach. To develop earlier end-points that would permit more rapid evaluation of treatment, we examined the time usually required to complete 3 major phases in the treatment of PV, i.e. control of disease activity, consolidation of treatment, and reaching the mid-point of the maintenance phase. The study was conducted in 37 consecutive patients with PV treated conventionally with prednisone in doses that were rapidly escalated in increments of 50% until disease activity was controlled (no new lesions and beginning healing of established lesions). That level was maintained until lesions were approximately 80% healed (end of consolidation phase). Steroid doses were then decreased by approximately 50% every two weeks. Adjuvants were used if there was a relative contraindication to steroids or if the disease flared when steroids were tapered. The end-points of the study were the time to control disease activity, to complete the consolidation phase, and to reduce peak doses of steroids by 50%; all measured from initiation of therapy at NYU. The median time to control disease activity was 14 (range 6-210) days, to the end of consolidation was 32 (range 12-245) days, and to reach the mid-point of maintenance was 53 (range 26-259) days. These end-points were reached more quickly in patients treated with higher (>90 mg/day) doses of steroids, but were unrelated to initial disease severity. These results provide previously unavailable data on the kinetics of the response of PV to treatment. They also provide several early end-points of response to therapy that can be used to compare more rapidly the effectiveness of new therapeutic modalities for PV.

0321

Non-invasive diagnosis of pigmented lesions

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This paper examines the different instruments used to aid dermatologists in establishing a diagnosis, how these benefit the patient, and improve follow-up care. The instruments range from simple loups and the variety of dermatoscopes (oil vs. non-oil based), to computer-assisted diagnostics and in-vivo microscopic imaging. Dermoscopy is a tool that most benefits those well-versed in its use. It can be used as a handheld alone, or with attachments such as photodermoscopes or videoscopes. Dermlite® has put out a new, non-oil dermatoscope, which is held above the skin, and may enhance visualization of vascular patterns. Newer computer-assisted instruments have been developed based upon various algorithms that may not necessarily use dermoscopy. Dermogenius® utilizes algorithms adopted from space technology, and supplements the ABCD rule by extra visualized dermoscopic features. Melafind® 100 employs multispectral imaging in order to examine different penetrations of the skin. MicroDERM® is a trained worldwide databank that operates through its archive of images. Vivascope 1000® is a non-invasive, in-vivo microscopic imaging technique that uses the naturally occurring refractive index variations in tissue without the use of stains. Some of these instruments may be costlier than others, but may require less formal training for the physician. Various instruments exist that aid the physician in non-invasive diagnosis of pigmented lesions. Their unique technologies allow for greater applications of the traditional methods of analysis. The new morphologic dimensions revealed, and the data they generate, are increasingly the subject of research to find more criteria for objective diagnosis.

0322

The difference in number and size of senile angiomas on skin subjected to irradiation for breast cancer compared to skin on the opposite non-irradiated breast

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Senile or cherry angiomas are part of the aging process and although sometimes they may be visually unsightly by their number and size, are not associated with any physiological or clinical impairments. To determine the effect of high dose x-irradiation on these lesions, the authors did a careful analysis of the number and largest size of cherry angiomas on the breasts of 207 women presenting for followup after previous high dose breast irradiation for unilateral breast cancer. One observer (MD) counted and measured the angiomas on the irradiated and opposite "control" breast of every patient. The 207 women ranged in age from 27-85 years (median 60 years) at the time of treatment. All irradiated breasts received a minimum of 5000 cGy in daily fractions of 180-200 cGy, five days per week, over 5-6 weeks. About 2/3 received adjuvant hormonal therapy and/or chemotherapy. The interval from radiotherapy to followup examination for angiomas ranged from 4-283 months (median 85). Twenty-six women (12.6%) had more angiomas on the irradiated breast skin and 48 women (23.2%) had an equal number on both breasts. One hundred thirty-three (64.3%) had fewer angiomas on the irradiated breast skin ($P<0.0005$ by the two tailed paired t-test). Angiomas on the irradiated breast skin were smaller ($P<0.0005$). Increasing age at treatment was the only factor predicting for a significant decrease in the number and size of ipsilateral versus contralateral angiomas. Thus, high doses to the skin received during irradiation of underlying malignancies can be associated with a decrease in the number and size of senile angiomas. Since these angiomas have no clinical significance, they do not require treatment.

0323

Selective and sustained impairment of γδ T cells and NK cells in patients with atopic dermatitis is driven by monocytes

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Although impaired type 1 responses have been demonstrated in patients with atopic dermatitis(AD), it remains undetermined whether innate immune cells capable of producing type 1 cytokines are also impaired and if so, whether their functions are intrinsically altered. We first compared the number of γδ+T cells and NK cells as well as adaptive immune cells such as αβ+T cells in the whole blood of AD patients and healthy controls. We found that whereas the numbers of αβ+T cells did not differ in these groups, those of γδ+T cells and NK cells were profoundly decreased in patients with AD. We next analyzed intracellular cytokine expression of proinflammatory (TNF-α), type-1 (IFN-γ) and type-2(IL-4) cytokines in these subsets after stimulation with PMA and ionomycin for various time intervals(0-2h, 2-4h and 4-6h) in these groups. Upon >2h stimulation, expression of TNF-α and IFN-γ, but not IL-4, in NK cells was significantly decreased in patients with AD as compared with controls (5.6% vs 9.6% in TNF-α; 19.2% vs 36.5% in IFN-γ, respectively). Furthermore, analysis of IFN-γexpression in γδ+T cells also showed a dramatic decrease in AD patients (35.0% vs 47.0%). In contrast, no significant differences were noted in between AD and controls in cytokine expression of adaptive immune cells. Surprisingly, removal of CD14+ monocytes from PBMC did lead to a dramatic recovery of the ability to produce IFN-γ and TNF-α of NK cells and γδ+T cells, but not αβ+T cells, to the levels more than that observed in controls. By contrast, these cytokine production in controls were not enhanced on the depletion of CD14+ monocytes. These observations indicate that a pronounced defect in the ability to produce TNF-α and IFN-γ of NK cells and γδ+T cells in AD patients is driven by monocytes.

0324

An open study comparing efficacy of 5% sodium L-ascorbyl-2-phosphate lotion versus 1% clindamycin phosphate lotion and vehicle lotion in the treatment of acne vulgaris

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We reported the efficacy of 5% sodium L-ascorbyl-2-phosphate lotion (5% APL) in the treatment of acne achieved by an open comparative study at the SID meeting in 2002. This time, we studied the clinical efficacy of 5% APL, 1% clindamycin phosphate lotion (1% CL) and vehicle (VE) for the treatment of acne vulgaris. One hundred patients were assigned to receive 5% APL (n=40), 1% CL (n=40) and vehicle (n=20) topically. Efficacy was assessed in 5 grades at the beginning and at 4, 8, and 12 weeks of treatment based on clinical photographs and the reduction rate of lesion counts. Patients were instructed to refrain from receiving treatment which has some possibility to have effects on acne lesions, such as administration of antibiotics, pill and sex hormone, metronidazole, retinoid and so on for 4 weeks prior to enrollment in the study. The number of patients who complied with the treatment protocol was 37 on 5% APL, 35 on 1% CL, 17 on VE. Efficacy rate for APL was 75.7%, Cl 62.9% and VE 17.6%. The efficacy of 5% APL was superior to that of 1% CL, which is widely prescribed in the treatment of acne vulgaris in the western countrieas. This data supports the hypothesis that 5% APL showed the excellent efficacy in the treatment of acne vulgaris, and therefore is better than 1% CL. This is the first demonstration of comparing efficacies between 5% APL, 1% CL and VE.

0325

Effect of narrowband UVB maintenance therapy on the duration of remission for psoriasis: a prospective randomized clinical trial

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Narrowband uvb phototherapy (nbuvb) is an effective treatment for psoriasis.Little is known about the duration of remission after nbuvb for psoriasis and the effect of maintenance therapy on the duration of remission.The aim of this study was to determine whether a two-month maintenance nbuvb effect the duration of remission.Patients were exposed to nbuvb three times weekly until initial clearing (defined by a 75% decrease of the PASI score).After initial clearing patients were randomly assigned to one of the 2 groups;namely maintenance group and no maintenance group.Patients designated to receive maintenance were treated further twice weekly for the first and once weekly for the next month.Patients in the no maintenance group received no further therapies.Both groups were followed for a minimum of 6 months.Of 50 patients enrolled, 46 (92%) cleared.Numbers of patients randomized to maintenance and no maintenance were 22 and 24, respectively.Six months after initial clearing 12 patients (55%) who received maintenance and 12 patients (50%) who did not receive maintenance were still clear.Life table analysis indicated that there was no statistically significant difference between maintenance and no maintenance groups for the probability of remaining in remission 6 months after initial clearing (p=0.76).During the first 2 months after initial clearing 2 patients in the maintenance group but non in the no maintenance group flared.The results of our study suggest that a two-month maintenance nbuvb fails to effect the duration of remission for psoriasis.However the observation that during the first 2 months after initial clearing 2 patients in the no maintenance group but no patients in the maintenance group flared encourage us to increase the number of patients in both groups for further investigation of the effect of maintenance nbuvb on the duration of remission.

0326

CD158k is a marker of the circulating tumor T cell clone in the blood of patients with Sezary syndrome

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CD158k belongs to the family of immunoglobulin-like inhibitory receptors. It is normally expressed on a minority of NK and CD3+CD8+ T lymphocytes. We have shown that CD158k is expressed by a major subset of CD4+ lymphocytes in patients with Sezary Syndrome. The aim of the present work was to study the correlation between the percentages of Sezary cells determined by cytomorphology, and of circulating CD158k+ lymphocytes in a larger group of patients, and to assess whether the CD158k+ phenotype was restricted to the malignant clonal population. We studied by flow cytometry the expression of CD4 and CD158k on lymphocytes of 35 patients with Sezary Syndrome and 6 erythrodermic controls. 12 Sezary patients had additional analyses during follow-up. We found a positive correlation between the percentages of CD158k+ lymphocytes and Sezary cells (r:0.58, p=0.0002), and between the percentages of CD4+CD158k+ lymphocytes and Sezary cells (r:0.61, p=0.0004). We also found a parallel decrease of the percentages of CD158k+ cells and Sezary cells in 6 patients who evolved into a complete or partial remission, and parallel increasing numbers of the percentages of CD158k+ cells and Sezary cells in 6 patients with a progressive disease. In a patient with more than 85% of Sezary cells and of circulating TCRVb2+ lymphocytes, we found that more than 85% of circulating lymphocytes were reactive with both anti-TCRVb2 and anti-CD158k mAbs. All CD158k+ lymphocytes were included in the TCRVb2+ cell population. Further, we isolated with a flow cytometer cell sorter the TCRVb2+CD158k+ cell population and showed that the sorted TCRVb2+ lymphocytes exhibit a unique TCRVb2 junctional region, indicating that CD158k receptor expression was restricted to the malignant clonal population. Therefore, CD158k expression could be a useful tool to identify the circulating tumor T-cell clone, for the staging and the follow-up of patients with a Sezary syndrome.

0327

Erythromelalgia: vasculopathy or neuropathy, or both? A prospective study of vascular and neurophysiologic studies in erythromelalgia

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To assess the frequency and type of vascular changes and neurologic abnormalities in patients with erythromelalgia. Prospective study of patients during periods without symptoms and with provoked symptoms. Setting: Tertiary referral center. Sixty-seven patients presenting with erythromelalgia from 1999 through 2001. Test nerve function and vascular function in patients without symptoms; test vascular function after provoking symptoms. In those patients in whom symptoms could be elicited, vascular function with and without symptoms was assessed by measurement of local skin temperature, laser Doppler flow, and transcutaneous oximetry. Neurologic assessment included electromyography, nerve conduction studies, and autonomic reflex screening (quantitative sudomotor axon reflex test, adrenergic function testing, heart rate response to deep breathing, and Valsalva ratio). Autonomic reflex screening was performed on 57 of the 67 patients (85.1%). Results were abnormal for 49 of the 57 (86.0%) patients who had autonomic reflex screening. Of these 57, 46 (80.7%) had abnormal quantitative sudomotor axon reflex test results, 14 (24.6%) had abnormal adrenergic function, and 15 (26.3%) had abnormal cardiovagal function. Electromyography and nerve conduction studies were performed in 24 of the 67 patients (35.8%). Of these 24, 14 (58.3%) had abnormal electromyographic results and 10 (41.7%) had abnormal nerve conduction study results. Vascular function studies, with and without symptoms, were performed in 13 of the 67 patients. During symptoms, the mean temperature of the toe skin increased by 7.8 C, and blood flow increased by 10.2-fold. Paradoxically, mean transcutaneous oximetry measurements did not change. This prospective study extends and confirms our previous observation that most patients with erythromelalgia have small fiber neuropathy, in addition to other forms of neuropathy.

0328

Analysis of predominant risk factors for developing melanoma among a cohort of patients at high-risk for developing melanoma

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The objective of our work was to analyze the predominant risk factors for developing melanoma (MM) in a cohort of high-risk patients and to evaluate the probability of developing MM in this cohort. Consecutive medical records from a dermatology office were reviewed. A total of 398 patients were selected who met the criteria of having (1) total cutaneous photograph as an aid for follow-up, (2) annual total cutaneous exam, and (3) at least six months of clinical follow-up. Risk factors, including age, sex, prior personal history of MM, family history of MM, personal history of non-melanoma skin cancer (NMSC), presence of classic atypical moles syndrome (CAMS, a triad of more than 100 moles, having atypical moles, and having one mole greater than 8mm in diameter), and presence of one or two of the CAMS triad, were estimated with univariate and multivariate analyses. Using the Kaplan-Meier method, the ten-year probability for developing MM was computed separately for patients who met the CAMS criteria (i.e., CAMS) and for those who did not meet the CAMS criteria (i.e., non-CAMS). Results: The cohort contained 237 CAMS and 161 non-CAMS patients. The mean follow-up period was 96.4 months. Univariate analysis identified age, prior personal histories of MM and history of NMSC as statistically significant risk factors. Multivariate analysis showed that only prior personal history of MM remained significant with a hazard ratio (H.R.) of 2.0 (adjusted 95% C.I 1.2-3.3). Hazard ratios for age and history of NMSC were no longer significant [adj. H.R. for age was 1.03 (95% C.I. 1.0-1.1); adj. H.R. for history of NMSC was 1.7 (95% C.I. 0.98-3.0)]. The cumulative 10-year risk for MM was 20% for the CAMS patients and 16% for the non-CAMS patients. The difference between the risks was not statistically significant. Conclusion: In this cohort of patients at high-risk for MM, a previous history of personal MM is the most significant risk factor for developing MM.

0329

Review of malignant melanoma in West China during 20 years

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Xijing Hospital and Xinan Hospital are the biggest hospitals in the North-West China and Southwest China respectively. We collected and analyzed the data of malignant melanoma (MM) in the two hospitals from 1981 to 2000. There were 353 cases of MM confirmed by pathological examination. Two hundred and fifty one cases were cutaneous malignant melanoma (CMM), and it was 0.063% of the total number biopsies, 0.578% of total number malignant tumor of the corresponding period. It was 0.044% and 0.073% of the total number biopsies in 1980s and 1990s respectively. The average growth rate was 3.4%. There were 231 cases that the lesion site could be learned from the available data. Among them, 47.0% were in the feet, 16.3% in the hands, 13.5% in the head and neck, 11.2% in the trunk. There were 202 cases that the causes have been mentioned in the available data. 25.7% were with the history of trauma in the primary site of MM, 19.3% originated from congenital small nevi. Among the 185 cases that with clinical and pathological data, 23.2% were in clinical stage1,21.1% in clinical stage2,32.4% in clinical stage 3, 17.8% in clinical stage 4,7.0% in Clark level 1,8.1% in Clark level 2, 15.1% in Clark level 3, 23.8% in Clark level 4, 19.5% in Clark level 5, 26.5% of them that the level of tumor infiltration was difficult to judge. Compared with the data of Europe and American, there are some characteristics of the CMM of West China: the incidence rate of CMM increases slowly; acral site is the predominant location of CMM; trauma is an important inducement of MM in acral site; congenital small nevi play an important role in the onset of MM.

0330

Adhesion molecule expression in vascular endothelial cells and cytokine production by macrophages in xanthoma

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To investigate the process of foam cell recruitment in xanthelasma palpebrarum, we examined endothelial adhesion molecule expression and cytokine production by macrophages using immunohistochemistry and *in situ* hybridization. ICAM-1 was constitutively expressed, but E-selectin and VCAM-1 were not expressed in endothelial cells of the vessels without cell infiltration. These endothelial cells did not produce MCP-1, IL-1β, TNF-α and TGF-β. At the sites of infiltration with macrophages, immature foam cells and lymphocytes, endothelial cells significantly expressed E-selectin and VCAM-1, but ICAM-1 was not expressed. Macrophages produced much more MCP-1 and IL-1β than endothelial cells. TNF-α production was extensive, but TGF-β production was small both in macrophages and endothelial cells. Within the foam cell nests, neither ICAM-1 nor E-selectin and VCAM-1 were expressed in endothelial cells. Foam cells and endothelial cells did not produce MCP-1, TNF-α, IL-1β and TGF-β. These findings suggest that E-selectin and VCAM-1 contribute to the adhesion of monocytes to endothelial cells, and MCP-1 of macrophage origin plays an important role as chemoattractant for monocyte/macrophages in xanthoma. In addition, IL-1β and TNF-α produced by macrophages should be potent cytokines in the foam cell infiltration. Mature foam cells, however, have lost such activities. TNF-α produced by endothelial cells might induce the adhesion molecule expression in an autocrine manner.

0331

Peroxiredoxin I is expressed in normal melanocytes and malignant melanoma in human

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Peroxiredoxin (Prx) is a kind of peroxidase to remove hydrogen peroxide, and has been identifed as at least 6 isotypes in mammalian tissue. In our previus study, Prx I, II, and III are revealed to be expressed in the skin. This study was aimed to evaluate the expression of Prx I in normal melanocytes and malignant melanomas in human. For immunohisotchemical study in melanomas, Prx I immunostaining was evaluated in paraffin-embedded samples from 17 Korean acral lentiginous melanomas (ALM) using an ABC technique. In relation to Prx I expression and the histologic levels of ALM, the superficial ALM was arbitrarily defined that tumor cells were invasive to the papillary dermis, and the deep ALM was defined that tumor cells reached to the reticular dermis. The intensity of Prx I staining was graded as +3 (stronger than epidermal staining), +2 (similar to epidermal staining), +1 (weaker than epidermal staining), and 0 (negative staining). In normal melanocytes, the ubiquitous expression of Prx I was identified by Western blot analysis, and the Prx I expression was localized to the cytoplasm by a confocal microscopy. The same pattern of Prx I expression was identified in several cell lines of malignant melanoma, such as G361, TMX13, A375SM, and A375P. In normal melanocytes and the melanoma cell lines, Prx I protein expression was induced from 24-48 hrs after hydrogen peroxide treatment at 5-10 micromolar concentration. Among 10 of the superficial ALM, 4 of (+1), 4 of (+2), and 2 of (+3) were graded, respectively. Among 7 of the deep ALM, 3 of (+1), 3 of (+2), and 2 of (+3) were graded, respectively. This study showed that Prx I is identified as a novel peroxidase in melanocytes and malignant melanoma in human. Further studies are warranted for the relation between the Prx I expression level and the tumor invasiveness or metastasis in malignant melanomas.

0332

Correlation of clinical severity and ELISA scores for NC16a domain of BP180 in bullous pemphigoid

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Titers of circulating autoantibodies detected by indirect immunofluorescence (IIF) have been used for the diagnosis and evaluation of disease activity in bullous pemphigoid (BP). In BP, the major pathogenic epitope is known to be the non-collagenous extracellular domain (NC16a) of the 180-kD transmembrane hemidesmosomal protein (BPAG2). Recently, an ELISA kit using the NC16a domain recombinant protein (BP180 ELISA kit) has become commercially available to measure the quantities of pathogenic autoantibodies circulating in BP patients. In the present study, we investigated the correlation of clinical severity and ELISA scores in clinically, histopathologically and immunohistopathologically typical BP patients. Ten patients with a typical form of BP and one refractory BP patient who died despite extensive treatment were included in this study. Antibody titers in sera from these patients were measured using BP180 ELISA kit and an analysis of ELISA scores and its correlaton with disease activity was performed. ELISA scores were significantly reduced after successful therapy with systemic corticosteroid, although IIF titers did not show apparent correlations in all the cases. In three patients, ELISA scores were under the cutoff value even from the onset of the disease and remained unchanged after successful treatment. In the patient with reflactory BP, ELISA scores also showed a fair correlation with disease course, although IIF titers did not clearly reflect disease activities. In conclusion, this commercially available kit more closely and correctly followed disease activities than the IIF titers. This BP ELISA system may be a useful tool to evaluate the disease activity and to assess the effectiveness of the treatment of BP patients in majority of BP patients whose pathogenic epitope exists in the NC16a domain.

0333

Pruritogenic mediators in psoriasis vulgaris

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To clarify the mechanism and mediators involved in the pruritus of psoriasis vulgaris, we quantitatively compared itch-associated factors in lesional skin from psoriatic patients versus skin without pruritus using a panel of histologic and immunohistologic parameters. When compared to psoriatic patients devoid of pruritus, lesionalskin from patients with pruritus showed the following characteristic features; a rich innervation both in the epidermis and in the papillary dermis, an increase in neuropeptide substance P-containing nerve fibers in perivascular areas, decreased expression of neutralendopeptidase in the epidermal basal layer as well as in the endothelia of blood vessels, many mast cell showing degranulating processes in the papillary dermis, a strong immunoreactivity for nerve growth factor throughout the entire epidermis and an increased nerve growth factor content in lesional skin homogenates, an increase in the expression of high-affinity receptors for nerve growth factor(Trk-A) in basal keratinocytes and in dermal nerves, an increased population of IL-2-immunoreactive lymphocytes, and a strong expression of E-selectin on vascular endothelial cells. A significant correlation was observed between the severity of pruritus and PGP9.5-immunoreactive intraepidermal nerve fibers, NGF-immunoreactive keratinocytes, expression of Trk-A in the epidermis and the density of immunoreactive vessels for E-selectin. These findings indicate that possible pruritogenic mediators in psoriatic lesional skin are neurogenic factors including innervation, neuropeptide substance P, neuropeptide-degrading enzymes and nerve growth factor, activated mast cells, one or more cytokines and endothelial-leukocyte adhesion molecules. These data document for the first time itch-related local markers in psoriasis, and suggest complex and multifactorial mechanisms of pruritus in the disease. These results provide the groundwork for further studies to evaluate the efficacy of anti-pruritic treatment for psoriatic patients.

0334

Three separate studies support the systemic safety of fluticasone propionate cream, ointment and lotion in the treatment of severe and extensive eczema in children as young as 3 months

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Three open, multicenter studies in paediatrics with extensive moderate to severe eczema evaluate the safety of FP 0.05% cream, FP 0.005% ointment and FP 0.05% lotion and investigated effects on hypothalamic pituitary adrenal (HPA)-axis function using the cosyntropin stimulation test (CST). In total, 135 children (82 were 3months - 3yr, 53 were >3-5yr) with extensive moderate to severe eczema (mean BSA involvement = 60-68%) applied FP twice daily for 3-4 weeks. There were no clinically significant differences in mean cortisol levels pre-and post-CST before or at end of, regular FP treatment. Pre-CST cortisols for all 3 FP formulations were similar before and after treatment. Before FP, the cortisols pre-CST for cream, ointment and lotion were (mean±sd) 14±7, 12±5, 13±6µg/dL, and post-CST were 31±7, 27±4, 35±6µg/dL, respectively. After FP, the cortisols pre-CST for cream, ointment and lotion were 12±7, 11±5, 12 ±6µg/dL and post-CST were 29±7, 25±4, 33±8µg/dL, respectively. Of the 120 children with end-treatment post-CST cortisols, 117 had normal responses (defined as 18µg/dL), only 3 had low values. One patient had samples taken at an inappropriate time of day and 2 had normal cortisols 7-12 days after cessation of therapy. No correlation was apparent between cortisols and available FP levels. Data from the 3 studies indicated that even extensive application of FP cream has minimal potential to cause atrophogenic effects. There were no withdrawals due to adverse events or clinically significant shifts in laboratory values. In paediatric patients, some as young as 3 months, FP in cream, ointment or lotion formulation, applied daily for up to 4 weeks, is safe for the treatment of severe and extensive eczema.

0335

Acute generalized exanthematous pustulosis: does it exist?

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Among pustular eruptions was defined in the early 80's, the Acute Generalized Exanthematous Pustulosis (AGEP). This reaction, mainly drug induced, is characterised by the acute onset of dozens to hundred of small, pinhead-sized non-follicular sterile pustules. They often arise on an edematous erythema, with accentuation of lesions in big folds. Recognition of this disease and its separation from acute pustular psoriasis is not worldwide admitted. During a 5 year-period, in a multinational case control study (EuroSCAR) based on an European detection network devoted to study severe cutaneous adverse reaction, we actively detected cases of acute pustular eruption. We recorded demographic data, previous medical history, clinical and histopathological description of the eruption, and recent drug intake. Cases were ascertained as definite or possible AGEP by an international expert committee, based on a scoring system taking into account morphology of the eruption, course of the disease and histopathology. We evaluated 150 potential community cases of acute exanthematous pustular eruption. Among those, 96 cases fulfilled criteria for AGEP. Mean age of patients was 55,5, (4-91) with a male/female sex-ratio of 0,8. Age repartition differs from that of Stevens Johnson syndrome and toxic epidermal necrolysis. The death rate was 4%. Erythema and pustules were widespread in 89% and 73% of patients with folds accentuation in 46% and 50% respectively. Recent herpes infection, HIV and other infections, autoimmune diseases, atopy, cancer and psoriasis were not associated or risk factors. Based on these prospectively collected data, we confirm the reality of this disease, with specific clinical pattern and evolution and no association to psoriasis.

0336

Pear-seed extract--a remarkable anti-aging active ingredient that significantly diminishes skin wrinkles and roughness: an *in vivo* study

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Compensating for age-related hormonal decline has recently become an essential approach to skin aging. Phytohormones are natural plant compounds with body-hormone-like activity. Consequently, enthusiasts have promoted phytohormones as the natural alternative to age-related hormonal decline. In this area, we developed a pear-seed extract rich in phytohormones, and we investigated its anti-aging properties in this double blind in vivo study. The study was conducted on twenty healthy women, age 40 to 62. The volunteers applied the cream formula with 2.5% of the extract, or placebo, on the eye zone area twice a day for 8 weeks. Skin wrinkles and surface roughness were evaluated clinically, using pictures, and through silicon replicas followed by statistical treatment using the matched paired student test. Silicon replica results showed that pear-seed extract application on the skin significantly reduced the number of skin wrinkles (-15.5%) and average wrinkle depth (-5.5%). Interestingly, highly significant results were found concerning the effect of the extract on reducing the total surface of wrinkles (-37,9%), total wrinkle length (-24.4%), and average wrinkle length (-13.6%). Moreover, analysis of skin roughness showed that application of the extract to the skin improved its texture and significantly reduced average roughness (Ra, - 5.7%). Furthermore, clinical examination of the volunteers skin following scoring method revealed a net visible improvement of skin surface and reduction of wrinkles by 18% in the extract-treated sides. These results demonstrate a remarkable and very significant effect of pear-seed extract on reducing skin wrinkles and decreasing skin roughness, which is of great interest in anti-aging skin care products.

0337

Reductions in circulating memory T cells correlate with clinical improvement and prolonged responses to alefacept in psoriasis

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Scientific and clinical evidence supports a critical role for memory T cells in the pathogenesis of psoriasis. This randomized, double-blind, placebo-controlled, parallel-group study examined the relationship between the pharmacodynamic and antipsoriatic effects of alefacept, a biologic agent that targets CD4+ and CD8+ memory T cells involved in psoriasis pathogenesis. Patients with chronic plaque psoriasis (n=553) were randomized to 1 of 3 cohorts: alefacept for 2 courses; alefacept in course 1, placebo in course 2; or placebo in course 1, alefacept in course 2. In each course, alefacept 7.5 mg or placebo was administered by IV bolus once weekly for 12 weeks followed by 12 weeks of observation. One or 2 courses of alefacept reduced CD4+ and CD8+ memory T cells, with negligible effects on the naive population. There was no evidence of a cumulative effect of alefacept on total lymphocyte or lymphocyte subset counts. Most patients had CD4+ counts above the lower limit of normal at 12 weeks after the last dose in the first course (88%) and second course (83%) of alefacept. In course 1, alefacept-treated patients with the largest decreases in memory T cells experienced the greatest reductions in disease activity (P<.001). The majority of the alefacept-induced reductions in the memory T-cell population preceded the peak improvement in Psoriasis Area Severity Index. Lasting remissions were evident in patients who responded to alefacept. The duration of clinical benefit was significantly longer among patients who had the greatest reduction in memory T cells relative to patients who experienced less of a reduction in memory T cells. In conclusion, 1 or 2 courses of IV alefacept selectively reduced circulating memory T cells and spared the naive T-cell population. The reductions in memory T cells were related to all measures of disease activity evaluated and the duration of response to therapy, suggesting that the memory T cell plays a key role in psoriasis. Results suggest that reduction of the pathogenic T-cell population can lead to prolonged remissions of psoriasis.

0338

Alefacept plus ultraviolet B light as combination therapy for psoriasis

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T cells play a critical role in the pathogenesis of psoriasis. Alefacept is a novel and selective biologic agent that blocks the natural interaction between LFA-3 on antigen-presenting cells and CD2 on T cells to inhibit T-cell activation and proliferation. In addition, alefacept engages FcγRIII receptors on accessory cells to reduce pathogenic memory T cells. Monotherapy with alefacept is well tolerated; remissions lasting 7-8 months have been demonstrated after 12 weeks of once-weekly dosing. Ultraviolet B (UVB) light is frequently used for the treatment of psoriasis. It directly targets rapidly proliferating keratinocytes and T cells in the skin and has a relatively rapid onset of action. The combination of alefacept and UVB light has the potential to provide synergistic efficacy and lower the cumulative dose of UVB light, thereby resulting in less photodamage and increased convenience. The combination of alefacept and either narrowband (NB) or broadband (BB) UVB light was evaluated in an open-label study of patients with chronic plaque psoriasis. Patients (approximately 60) were enrolled at 2 sites: 1 in France (NB UVB) and 1 in the US (BB UVB). All patients received alefacept 15 mg IM once weekly for 12 weeks. Patients were randomized (1:1:1) to receive either no UVB light, UVB light 3 times/week until clear or for up to 6 weeks, or UVB light 3 times/week until clear or for up to 12 weeks. An observation period of 12 weeks followed the treatment period. The primary objective was to determine the safety and tolerability of combination therapy, primarily via assessments of adverse events, including infections, changes in minimum erythematous dose, and skin irritation. The efficacy of combination therapy was determined by assessments of Psoriasis Area Severity Index and physician and patient global evaluations. Results will be presented.

0339

Repeated courses of IM alefacept in psoriasis: rationale and design of an international study that mimics the clinical practice setting

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Psoriasis is a chronic, relapsing skin disease that typically requires life-long treatment. Alefacept is a novel biologic agent that improves psoriasis by selectively targeting the T cells involved in the disease pathogenesis. It has been shown to have an encouraging clinical profile in multiple randomized, placebo-controlled studies and retreatment trials. To understand the best way to manage existing therapies during a course of alefacept, an international study is being conducted to mimic the clinical practice setting. Approximately 400 patients with chronic plaque psoriasis will be enrolled in this open-label study in which topical treatments, narrowband or broadband ultraviolet B light, systemic retinoids, and prednisone will be permitted as concomitant therapies. Cyclosporine and methotrexate will be tapered over the first 6 weeks of alefacept therapy. Patients must be at least 16 years of age, require systemic therapy, have normal CD4+ lymphocyte counts (at least 300 cells/mm3 if on stable dose of prednisone), and be naive to alefacept therapy. In each course, alefacept 15 mg will be administered once weekly by IM injection for 12 weeks followed by 12 weeks of observation. Patients may receive up to 3 treatment courses. The primary objective is to evaluate the safety of repeat courses of alefacept, as assessed by adverse events, selected blood chemistries, vital signs, and physical examinations. Other prespecified objectives are to determine the efficacy of initial and repeat courses of alefacept and the duration of response after the first 2 courses. Circulating total and CD4+ and CD8+ lymphocyte counts will be determined. Physician Global Assessment will be performed at baseline and at multiple times during the observation period.

0340

Electronic monitoring of medication adherence in skin disease clinical studies: results of a pilot study

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Medication non-adherence is a financial burden to the medical system and is understudied in dermatology. Currently all studies involve self-report, pill counts or medication tube weights which have been shown to be inaccurate. The purpose of this study was to examine medication adherence to topical therapy using electronic monitors. Topical salicylic acid was administered in a jar with an electronic cap (MEMS cap) that monitors of usage of medication to ten subjects participating a clinical study for localized psoriasis. The MEMS cap data were compared to data recorded by patients on dosing calendars. Eight out of 10 subjects reported more medication use on their logs than was actually recorded by the MEMS caps. Self reported use of topical medications is inaccurate and much of the failure of topical medications is likely due to medication non-adherence. Electronic medication monitors, like the MEMS cap, may be integrated into future clinical trials to accurately show response vs. non-response vs. non-adherence to investigational therapies.

0341

Use of topical tacrolimus in combination with 6% salicylic acid for treatment of psoriasis: results of a double blind right to left trial

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Topical tacrolimus is an effective anti-inflammatory agent in atopic dermatitis but appears less effective on thick, scaly psoriasis lesions. The purpose of this study is to assess whether combined use with a penetration enhancer is effective. Twenty-three subjects with generally symmetric psoriasis were randomized to salicylic acid/placebo vs. salicylic acid/topical tacrolimus in this left-right comparison study. Treatment was given for eight weeks. The primary outcome was the change in the sum of erythema, scale and thickness scores from baseline to end of treatment. The medications were well tolerated. Significant improvement was found in subjects with moderate to good adherence. Fifty percent of the subjects completing the study requested a prescription for tacrolimus. The preliminary data suggest that combination therapy using salicylic acid with topical tacrolimus may increase the effectiveness of the topical tacrolimus in the treatment of psoriasis.

0342

Withdrawn.

0343

Alefacept is efficacious for treatment of psoriasis regardless of response to prior therapy or disease severity: pooled analyses of phase 3 trials

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The efficacy and safety of alefacept have been evaluated in 2 randomized, double-blind, placebo-controlled phase 3 trials including >1000 patients with chronic plaque psoriasis. Alefacept 7.5 mg IV, alefacept 10 mg IM, alefacept 15 mg IM, or placebo were administered oce weekly for 12 weeks followed by 12 weeks of observation. In both studies, almost 80% of patients had received prior systemic or phototherapy. Baseline disease severity was similar for the 2 trials. Across all treatment groups, median body surface area (BSA) involvement ranged from 20-24% and median Psoriasis Area Severity Index (PASI) from 13.2-15.9. In the pooled alefacept group (n=706), a 75% or greater reduction in PASI was achieved at any time during treatment or follow-up by 34% of patients who reported no change or worsening of disease during prior therapy, 26% of patients who reported improvement on prior therapy, and 34% of patients who had not received prior systemic or phototherapy. Corresponding percentages in the placebo group (n=354) were 12%, 9%, and 12% (all P<=0.002 vs alefacept). A 50% or greater reduction in PASI at any time during treatment or follow-up was achieved by 60% of alefacept patients who reported no change/worsening with prior systemic or phototherapy, 52% of those who had had improvement, and 60% of those with no previous exposure to systemic or phototherapy. In the placebo group, 23%, 29%, and 36% of patients, respectively, met this criterion (all P<=.001 vs alefacept). For patients with BSA >30%, a 75% or greater reduction in PASI was achieved by 25% of alefacept-treated patients (n=225) compared with 10% of placebo-treated patients (n=108). The corresponding response rates for those with BSA of 30% or lower were 31% for alefacept (n=481) and 10% for placebo (n=245). In conclusion, alefacept was efficacious irrespective of response to prior systemic or phototherapy and regardless of disease severity.

0344

The usefulness of a BP180 NC16A-domain ELISA in the serodiagnosis of pemphigoid gestationis and in differentiating between pemphigoid gestationis and pruritic urticarial papules and plaques of pregnancy

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Pemphigoid gestationis (PG) is a rare pregnancy associated subepidermal immunobullous disease, targeting hemidesmosomal proteins. Clinically, PG can resemble PUPPP and accurate differentiation between these two pruritic pregnancy related dermatoses has important implications for foetal and maternal prognosis. Epitope mapping studies show that autoantibodies in 90% of PG sera target a well-defined membrane proximal NC16a domain of BP180 (Morrison et al. J Clin Invest 1988;81:2023-26). The purpose of this study was to examine the usefulness of a commercially available NC16a-domain ELISA in the serodiagnosis of PG, and its diagnostic value in differentiating PG from PUPPP. Sera from 410 female subjects, consisting of PUPPP (n=164), normal female healthy volunteers (NHV), 18-50 years old (n=164) and pre-treatment PG patients (n=82) were assayed in duplicate according to the manufactures instructions. Receiver-operating-characteristic (ROC) analyses were performed to determine a cut-off value for the diagnosis of PG and differentiation from PUPPP. The average ages of PG, PUPP, and NHV were 32, 31 and 33, respectively. A cut-off value of 18 units is associated with a specificity and sensitivity of 99% (326/328) and 88% (72/82), respectively. The average coefficient of variation was 4.3%. None of the NHV and two of the PUPPP sera results were greater than 18. False positive and false negative results were further investigated by western immunoblotting studies using human epidermal extract and recombinant polypeptides encompassing the entire BP180 extracellular domain. The NC16a ELISA has a sensitivity consistent with the finding that 10% of PG sera react with BMZ epitopes outside the NC16a domain and is highly specific in differentiating PG from PUPPP.

0345

Prognostic factor analysis for plaque psoriasis

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Many patients with plaque psoriasis have good prognosis. However, some patients have refractory disease with poor response to drug therapy. Little attention has been given to the prognostic factors which determine the progression of psoriasis. The purpose of this study was to explore the prognostic factors and develop an index to assess the risk for progression of the disease. In March 2001, data including patients' clinical characteristics and disease condition at the time of diagnosis as well as the current disease condition was collected for 169 psoriasis patients who visited Kurashiki Central Hospital from March 1991 to March 1996 (median follow-up time 11.6 years). Ten factors of patients' clinical characteristics at diagnosis (age, sex, body mass index, the state of alcohol drinking and smoking, and the presence of concomitant diseases including joint pain, hypertension, hyperlipemia, diabetes, and the others; all were binary) were analyzed using logistic regression to associate the factors with the probability of progression or no change of the disease following diagnosis. Age (Odds ratio (OR) 0.271; 95% confidence interval (CI) 0.102-0.723; p=.0091), sex (OR 2.670; 95%CI 1.018-7.001; p=.0459) and body mass index (OR 2.735; 95%CI 1.009-7.415; p=.0480) had significant effects. Based on the result, an index was developed by using these three factors as follows; age < 40 years (1 point), male (1 point), and body mass index $\propto$ 25 (1 point). The sum of these three binary factors formed four subgroups (0-3 points). The rate of progression or no change of the disease for each subgroup (0, 1, 2 and 3) was 5%, 28%, 42% and 100% respectively. This prognostic index should be validated in future studies. The developed index may be useful in the design of future clinical trials for psoriasis and in the selection of appropriate therapeutic approaches for individual patients.

0346

Cutaneous squamous cell carcinoma incidence is not increased in rheumatoid arthritis patients receiving etanercept

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A recent report described 7 cases of cutaneous squamous cell carcinoma (SCC) in patients treated for rheumatoid arthritis (RA) with etanercept (ENBREL®) (J Am Acad Dermatol 2001, 45:953-6). Treatment with immunosuppressive agents such as cyclosporine has been associated with increased incidence of SCC, however data from animal models and clinical trials suggest that TNF inhibition does not increase malignancy rates. Because cancer surveillance is a central element in the etanercept pharmacovigilance program, we explored the potential association between etanercept therapy and SCC in RA patients, calculating observed and expected incidences of SCC from pooled etanercept clinical trials and long-term extension studies. Through 04/02, 1442 RA study patients received etanercept therapy with 4044 patient-years of cumulative exposure. Median and maximal durations of exposure were 3.7 and 5.7 years, respectively. The NCI Surveillance, Epidemiology, and End Results database (SEER) was used to determine expected number of all malignancy types for the general population. SEER does not provide incidence figures for non-melanoma skin cancers, so we used two published reports (from northern and southern US latitudes) to calculate incidence of SCC in an age- and sex-matched cohort from the general US population. The incidence of all cancers in etanercept-treated patients was less than expected. An age- and sex-matched population would be expected to have 6 to 13 cases of SCC in the interval of observation. However, only 4 cases were observed. Epidemiologic analysis of clinical trials provides additional perspective on the cancer risk associated with etanercept therapy. In long-term clinical studies with over 4000 patient-years of exposure, RA patients on etanercept therapy did not have an increased rate of malignancies. Importantly, the rate of SCC was well below the expected rate.

0347

Effect of short-contact topical tretinoin therapy on lower extremity ulcers in diabetic patients

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Topical tretinoin is a potential therapy to enhance the healing of wounds, given its properties of increasing granulation tissue, collagen synthesis, angiogenesis, and fibroplasia. The purpose of the current study was to test the hypothesis that topical tretinoin can improve healing of lower extremity ulcers in diabetic patients. This was a prospective, randomized, double-blind, placebo-controlled study involving 24 patient volunteers from the Diabetic Foot Clinic. Patients were divided into two treatment groups- treatment with topical tretinoin 0.05% solution and treatment with control normal saline solution. Exclusion criteria were: unable to give informed consent, having a known bleeding disorder, pregnancy at time of enrollment, patients with infected ulcers, and patients with lower extremity ulcers due to large arterial disease. At the initial visit, photographs were taken for measurements of the initial size of all lower extremity ulcers. Patients were then randomly assigned to one of the two treatment groups. Solution was applied direct to the wound bed and left in contact for a maximum of 10 minutes daily, then rinsed off with normal saline. This was done once daily for 4 weeks. Photographs and measurements of ulcer size were taken every 2 weeks after starting the assigned treatment, for a maximum of 16 weeks or until complete healing of the ulcer occurred, whichever came first. Tretinoin 0.05% solution reduced the mean time to ulcer healing relative to control normal saline solution ($p < 0.05$). For superficial ulcers (<1mm depth), subjects in both groups achieved complete healing by the end of the study. For deeper ulcers, 6/7 patients in the tretinoin treatment group achieved complete healing at 16 weeks as compared to1/4 patients in the control group. Patients continued to receive standard care for their ulcer. Topical tretinoin can improve the process of wound healing for diabetic ulcers. It decreases time to healing, without significant irritation. It has potential utility as an adjuvant therapy to current methods of treating diabetic foot ulcers.

0348

Interleukin-18 in atopic dermatitis and psoriasis

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Interleukin (IL)-18 is a proinflammatory cytokine known to be produced by keratinocytes and to have a broad role in inflammatory responses. Since IL-18 may contribute to both Th1 type responses and Th2 type responses depending on the circumstances they involved, enhanced expression of IL-18 had been reported both in atopic dermatitis and psoriasis. To elucidate the possible role played by IL-18 in atopic dermatitis and/or psoriasis, IL-18 and IL-18 receptor (R) were stained by a standard immunohistochemical technique using anti-IL-18 and anti-IL-18R arpha chain monoclonal antibodies in parafin-embeded tissue samples. In the lesional skin of atopic dermatitis and also in psoriasis, the staining of IL-18 was significantly enhanced in all the layers of epidermis compaired to normal control, while IL-18 R was also enhanced but localized in keratinocytes in subcorneal layer. Serum IL-18 levels were measured by ELISA. In comparison to normal control group, both patients with atopic dermatitis and psoriasis were found to exbit increased IL-18 levels, which appeared to be correlated with disease severity. These results may suggest that IL-18 is not the primary pathogenic cytokine either in atopic dermatitis or in psoriasis. However, IL-18 may be involeved in the cutaneous inflammatory process and/or keratinocyte changes, such as acanthotic epidermis seen both in atopic dermatitis and psoriasis.

0349

Fiberoptic confocal imaging of tbe skin

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Fiber optic confocal imaging (FOCI) is a new technology for imaging the skin. FOCI uses visible light (488 nM) to non-invasively produce high magnification (1000x) fluorescent images in-vivo. In contrast, most previous work with in-vivo confocal microscopy has used reflectance mode. A fiber optic laser scanning instrument, the Stratum (Optiscan, Melbourne Australia), was used to image a number of skin disorders as well as normal skin. Patients who were scheduled to have a skin biopsy were asked to participate in the study (FMLH IRB#02-111, MCW HHRC #187-02). Fluorescein, 0.2% was used as a fluorophore and 0.2 cc was injected in the high dermis at the site to be imaged. The site was then three dimensionally imaged to a depth of 200 micrometers. Controls included imaging adjacent, non-diseased tissue from the same patient and imaging the diseased area without the addition of dye. Following FOCI, the patient proceeded with a biopsy and FOCI images were compared to standard histologic images. No autofluorescence of the skin was found with the Stratum. FOCI provided clear images of the epidermis and the papillary dermis. The stratum corneum, stratum granulosum, stratum spinosum, stratum basale and papillary dermis could be identified by their specific morphologic features. The clarity of FOCI images also revealed micro-anatomical structures, such as eccrine ostia and the capillary plexus of the papillary dermis. Imaging of the autoimmune sub-epidermal blistering disease, bullous pemphigoid, allowed identification and localization of the blister cavity at the basement membrane zone. In contrast, pemphigus foliaceus, characterized by autoantibody binding to desmoglein 1 on the epidermal keratinocyte surface and subcorneal blisters, demonstrated widening of the intercellular spaces and a superficial blister cavity on FOCI. These studies indicate that FOCI can be used to identify specific morphologic features of normal and diseased skin.

0350

Age-related changes of skin microtopography in Caucasian and Japanese women

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The skin surface is characterized by a special relief which reflects the three-dimensional organization of the deeper layers and the functional status of the skin. The aim of this study was to compare changes of skin microtopography with age in Caucasian and Japanese women. Two studies were performed simultaneously during winter time in Paris and Sendai on 356 Caucasian and 120 Japanese healthy women respectively, aged from 20 to 80 years. Skin replicas were obtained from the volar forearm and analysed by interferometry. This analysis yielded to 12 standard "global parameters" generally used to describe surface topography and 13 "local parameters" including distribution of the lines according to their orientation, depth of lines and an anisotropy index. Statistical analyses included principal component analysis in order to visualize the links between the 25 parameters, and Pearson correlation coefficient to study the link with age for each parameter in both populations. All but one of the "global parameters" were found to be positively correlated with age. Moreover, Caucasian and Japanese women showed an increase in the "density of lines" >60µm of depth and a decrease of the "density of lines" <60µm with age. This phenomenon was found to be more pronounced and to appear at a younger age in Caucasians. Concerning the age-related changes in the "density of lines" according to their orientation, no significant change was found in the Japanese, whereas important changes were found in Caucasians, resulting to an important increase in skin anisotropy with age. Our results demonstrate that in both Caucasian and Japanese women important changes of the skin microtopography occur with age and that these changes were found to be more pronounced and to appear at a younger age in Caucasians.

0351

UV-free phototherapy: development and first clinical results

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Phototherapy with ultraviolet (UV) radiation is of benefit for patients with atopic dermatitis (AD). Since UV radiation is a complete carcinogen, UV therapy is usually not used for the longterm treatment of children and young adults and in combination with immunosuppressants. Phototherapy with UVA-1 (340-400 nm) radiation induces apoptosis in skin infiltrating T-cells via the generation of singlet oxygen. In the present study we have asked whether phototherapy with visible light, which according to current knowledge does not cause skin cancer but can generate singlet oxygen, can be used for the treatment of AD. For this purpose a novel large-area, laser-like photonic partial body irradiation device (DermoDyne) has been developed. Over 40% of the radiation is being emitted between 400-500 nm, with 26% of the spectral energy between 400-450 nm. Emission in the UV range is 1.5% between 380-400 nm, 0.1% between 360-380 nm, 0.01% between 340-360 nm and no emission below 340 nm. The residual UVA-1 emission was less than 1 J/cm2 per treatment, and thus, by definition (ICNIRP/IRPA), DermoDyne therapy is UV-free. Patients with atopic hand and feet eczema (n=10) were first treated for 2 weeks (3x/week) with a sham irradiation device emitting the identical spectrum but at a significantly reduced fluence (factor 105 less), after which they were subjected to DermoDyne treatment (3x/week) for another 4 weeks. Sham irradiation did not cause any improvement, as assessed by means of the DASI score, and in fact, one patient dropped out because of worsening of eczema. In contrast, the 9 remaining patients showed a significant improvement after 4 DermoDyne treatments (DASI, before 34.8; after 4 irradiations: 23.33; $p<0.0002$), with further significant improvement after another 8 irradiations (DASI, after 12 irradiations: 7.44; $p< 0.0001$). This study demonstrates the first successful use of UV-free phototherapy.

0352

Celecoxib prevention of BCCs in basal cell nevus syndrome patients

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Cancer chemoprevention studies in humans are difficult to conduct even in genetically-susceptible populations because the tumor incidence generally is so low that either large numbers of subjects must be studied or surrogate end points must be substituted for the actual cancer of interest. Patients with the basal cell nevus syndrome (BCNS) represent a uniquely attractive population for chemoprevention studies because they develop multiple tumors annually, thus permitting the rapid accumulation of statistically significant data in a small cohort. We have initiated a clinical trial to assess the efficacy of an oral cyclooxygenase-2 inhibitor in preventing BCCs in this population. The study design is a randomized double-blind trial of subjects receiving placebo or celecoxib, 200 mg bid, for two years. Patients are seen at sites in New York, Cleveland, San Francisco, or Los Angeles every three months, and their tumors are treated by their local primary skin care physician (PSCP) by whatever technique seems best suited to that tumor. The primary end point of the study is the number of BCCs of $\propto$ 3 mm diameter developing during the two years of therapy, and the enumeration is based on the records of the PSCPs, visits to the sites, and patient histories. We now have enrolled 40 of the projected 60 subjects, and they have been in the study for up to 18 months. No study-related adverse events have been detected, and only one subject has had to be eliminated for non-compliance. Among the obstacles to full enrollment are the rarity of BCNS, and the reluctance of patients to participate in a trial in which they may receive only placebo, to discontinue previously prescribed NSAIDs, and to come for visits every three months despite travel reimbursement. Nonetheless this population is very important for chemoprevention studies, and our involvement with BCNS patients has shown how committed they are to finding preventive approaches to their multiple health problems.

0353

Treatment of moderate-to-severe psoriasis with human monoclonal antibody to human IL-12

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The safety and efficacy of single, ascending, intravenous doses of a human monoclonal antibody to human IL-12 was assessed in patients with moderate-to-severe psoriasis. In this phase I, nonrandomized, open-label, multicenter study patients received a single intravenous infusion of a human monoclonal antibody to human IL-12. Eighteen patients with at least 3% body surface area involvement were sequentially enrolled into ascending groups (0.1 mg/kg, 0.3 mg/kg, 1 mg/kg, and 5 mg/kg). Six millimeter punch biopsies were performed the day prior to infusion, at 48 hours and 2 weeks post-infusion and analyzed using quantitative Taqman PCR to compare levels of cutaneous cytokine mRNA. Average Psoriasis Area and Severity Index (PASI), Physician's Global Assessment (PGA) and blood work were obtained during a 16 week period. PASI scores were reduced 56% by week 8 and 50% at week 16 among patients in the lower dose groups (0.1 mg/kg and 0.3 mg/kg, n = 8). In the higher dose groups (1.0 mg/kg, 5 mg/kg, n = 10), PASI scores were reduced by 75% by week 8 and 87% at week 16. Treatment was well tolerated with no drug related serious adverse effects. Absolute total CD4 counts fell to below 400 in 2 patients for less than 5 days. At 72 hours following study drug administration, CD16+56 counts decreased by >20% from baseline in 8 of 17 patients evaluated. Quantitative assessment of cutaneous cytokine mRNA showed IFNγlevels were significantly reduced at 2 weeks post infusion. In summary, a single intravenous infusion of a human monoclonal antibody to human IL-12 was well tolerated and induced significant clinical improvement in this open-label, phase I study.

0354

Correlation between the presence of C5-9 and immunoglobulins in skin and their clinical relevance

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Past evidence suggests direct immunofluorescence (DIF) could be a very useful tool in clinical medicine. In the past two decades, presence of C5-9 in dermoepidermal junction (DEJ) has been suggested to be unique for dermatomyositis. This study was designed to search for the correlation between C5-9 deposition and immunoglobulins in skin, and its relevance in clinical medicine. Skin biopsy specimens were transported to laboratory in Michel's solution, and processed for DIF and indirect IF (IIF) for C5-9 deposition. A total of 996 samples from patients was included. Among them, 129 were positive for IgG, while 47 for IgM, 49 for IgA, 156 for C3, and 183 for C5-9. 61 were lupus band test positive, and 58 of them had LE. 3 others had granuloma faciale and actinic keratosis. All those 61 cases were also positive for C5-9 in granular staining pattern along DEJ. There were 51 bullous pemphigoid (BP) cases with IgG and C3 staining. 4 of them also had IgM and/or IgA along BMZ. 50 of those 51 BP cases were positive for C5-9 along BMZ. 7 pemphigus patients were included, and all of them had igG, C3 and C5-9 staining along IC space of skin. 12 cases of dermatitis herpetiformis were included, and 4 of them also had IgG, IgM, C3 as well as C5-9 staining along DEJ. There were 16 patients with prodromal BP. 5 of them had IgG, C3 and C5-9 linear staining along BMZ. 9 other patients had C3 and C5-9 only. While the remaing 2 patients with prodromal BP had granular C5-9 staining along BMZ. Out of 996 specimens, 21 had granular C5-9 staining along DEJ only. The diagnosis of those 21 cases included 2 for prodromal BP, 10 for early LE, 4 for lichenoid vacuolar interface dermatitis and others such as drug reaction, allergic dermal reaction or urticarial vasculitis. In conclusion, there is strong correlation between the presence of C3 and C5-9 staining among those patients with definite diagnosis. Presence of only C5-9 staining along DEJ might suggest the early stage of BP or LE, if there is appropriate clinical setting.

0355

A randomized comparative study of Psoriasis Area and Severity Index, Psoriasis Global Assessment, and Lattice System-Global Psoriasis Score in rating psoriasis

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Accurate and reliable assessment of disease severity in psoriasis is essential in clinical research to document response to treatment. The reliability of current clinical outcome measures is uncertain. Therefore, we examined the relative variation in two commonly used outcome measures (Psoriasis Area and Severity Index [PASI], and Psoriasis Global Assessment [PGA]), and in a newer measure, Lattice System Global Psoriasis Score (LS-GPS), when used for rating psoriasis severity. This was a single center, randomized evaluation of 35 patients with psoriasis involving 17 investigators who were either experienced (53%; 9/17) or inexperienced (47%; 8/17) in using PASI and PGA. Each investigator evaluated each patient with each rating system in the morning and again in the afternoon. Using an analysis of variance model, we assessed the variation in the results of scoring psoriasis between (inter-rater) and within (intra-rater) investigators. The PASI, PGA, and LS-GPS were highly correlated (r>0.8 for all comparisons). PGA had the lowest intra-rater variation (sigma=0.2), followed by LS-GPS (sigma=0.5) and PASI (sigma=2.5). LS-GPS provided the most scores that were exactly the same at the two evaluations (reliability coefficients were 36% and 49% lower for PGA and PASI, respectively). As expected, there was higher inter-rater variability in assessing the same patient than that of the same rater over time. Inter-rater variation was low for PGA (sigma=1.2) and LS-GPS (sigma=1.7), and highest for PASI (sigma=8.8). Experience was beneficial in reducing variation in PASI scores but was not required with PGA or LS-GPS. PASI, PGA, and LS-GPS are reliable measures for assessing psoriasis. Because the numerical PASI may not be used by the US FDA, systems like the PGA and LS-GPS that provide word-based, intuitive ratings of psoriasis, and do not require experience, are useful.

0356

Severe hand dermatitis is responsive to topical bexarotene 1% gel

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Hand dermatitis is a common and therapeutically challenging clinical problem. Topical bexarotene gel 1%, an RXR selective retinoid in an alcohol gel, was tested in 55 patients with chronic severe hand dermatitis. Patients were randomly assigned to one of three treatment groups: bexarotene gel monotherapy or bexarotene gel in combination with either mometasone or with hydrocortisone ointment. Bexarotene gel applications were escalated every two weeks from QOD to TID; steroids were applied BID. Overall results (completed in 51 of 55 patients) showed 50% improvement in 67% of patients and 90% improvement in 35% of patients by physician assessments. Patients treated with bexarotene gel monotherapy had higher response rates of 79% at 50% improvement and 39% at 90% improvement, similar to results with the combination of mometasone and bexarotene gel. The combination with hydrocortisone ointment had a lower response rate than bexarotene gel alone, perhaps due to formulation interactions. Responses were confirmed at multiple evaluations. Both dorsal and palmar surfaces responded to treatment. Responses were confirmed by an independent computer-generated hand eczema area severity index. The median time for response was 5 weeks at 50% improvement and 10 weeks at 90% improvement. Responses were durable through the four-week follow-up. Bexarotene gel treatment reached BID in 87% and TID in 73% of patients. Adverse events included occasional mild/moderate pain/burning in 11%, and local irritation in 30% of patients. Bexarotene gel monotherapy had the lowest incidence of pain and fewer adverse events. Three patient withdrawals were related to adverse events, one of which was related to bexarotene. Local adverse events were minimized by confining bexarotene gel only to dermatitis lesions. The promising response rate with limited side effects suggests bexarotene gel may have therapeutic potential for hand dermatitis.

0357

Correlation of National Psoriasis Foundation score components with quality of life measures in psoriasis

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Psoriasis has a profoundly negative impact on an individuals? Quality of Life (QoL). Despite this, the Psoriasis Area and Severity Index (PASI) score, which has been shown to have a poor correlation to quality of life measures, remains the most commonly used measurement tool in clinical trials. While the PASI score is purely an objective measurement of the character and amount of psoriasis, an alternative assessment tool, the National Psoriasis Foundation (NPF) score, includes two aspects of subjective patient input (the patient-reported itch and the patient-reported global assessment) in addition to three other objective measures (physician global assessment, change in body surface area of psoriasis, and target plaque induration). The intent of this study is to: 1) measure correlation between the patient-reported parts of the NPF score and validated QoL measures and 2) introduce and run correlations on two newly designed measures, a five point desired improvement tool and a 100 point life quality assessment (LQA) scale. To perform this analysis, we designed a questionnaire to be given at baseline and follow-up visits to all adult psoriasis patients presenting to the University of Utah Dermatology clinics that is composed of: 1) the Dermatology Life Quality Index (DLQI), 2) Psoriasis Quality of Life Index (PQLI), 3) patient reported itch, 4) patient global assessment, 5) the desired improvement tool, and 6) the LQA scale. Study design includes correlation of the above components at baseline and correlation of their change over time on approximately 200 patients. Correlation coefficients on the first 50 patients follow: DLQI and PQLI (r=0.99), DLQI, DLQI and LQA scale (r=0.58), DLQI and the desired improvement tool (r=0.29), and DLQI and patient itch + global + LQA (r=0.57). The correlation between the QoL measures and the patient reported portions of the NPF, will likely increase when analyzed as change over time and indicates that the NPF score may be an easy and consistent assessment of the impact of psoriasis on a patients? life.

0358

Primary cutaneous peripheral T cell lymphoma, unspecified: favorable outcome for patients with localized disease regardless of cell morphology

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Peripheral T-cell lymphoma, unspecified (PTCL, NOS) is a WHO classification for a heterogeneous group of T-cell lymphomas that are not identified as recognizable subtypes. To study the clinicopathologic features and outcome, we present a retrospective analysis of 12 pts evaluated at Stanford w/primary cutaneous PTCL, NOS (nonepidermotropic, CD3+, CD20-, CD30-, CD56-). The M:F was 5:1. IPIs ≤ 1. Age range was 24-87, w/median of 53. Nine pts (75%) had localized dz (8 solitary). Three pts (25%) had generalized dz. Of 9 pts w/localized dz, 5 received local XRT, 2 systemic chemo+(EXC or XRT), 2 observed w/o tx. Two pts w/generalized dz received multiple systemic tx, 1 observed w/o tx. Four-year overall survival for localized vs generalized dz was 88% vs 50%. Four-year event-free-survival for localized vs generalized dz was 88% vs 0. Eighty-nine percent of pts w/localized dz had no evidence of dz at last f/u. Follow-up ranged from 5-114 mos, w/median f/u of 39 mos. Three pts died (25%); 2 had generalized dz, 1 had localized dz w/locally invasive bone involvement at presentation. Two dermatopathologists independently reviewed and confirmed the pathology for all 12 pts. Using EORTC histologic criteria, 4 patients presented w/large T-cell morphology, all w/localized dz, only 1 progressed/died. Two of 8 pts w/small/medium cell morphology (generalized dz) progressed/died. The initial interobserver agreement for histologic subtype was only 75%. Lack of agreement was due to difficulties in determining the proportions of neoplastic vs reactive T-cells and the accurate percentage of large neoplastic T-cells. These difficulties call into question the utility of EORTC histologic subtyping for prognostic and therapeutic purposes. In conclusion, PTCL, NOS, localized, appears to have a favorable outcome and localized treatments are appropriate. PTCL, NOS, generalized, may warrant more aggressive tx w/systemic therapies.

0359

Kappa opioid system is itch suppressive: a new approach for itch treatment

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Although itch is common symptom, severe itch is difficult to manage and its mechanism is not yet to be fully elucidated. Recently, the mu opioid system has been suggested as being one of the major pathways for itch sensation. We examined endogenous opioid systems in hemodialysis patients who suffer from uremic pruritus and found that kappa opioid system contributes itch control as well as mu opioid system. In itchy patients, serum mu opioid peptide (beta-endorphin) and its receptor on peripheral lymphocyte were relatively expressed more than kappa opioid peptide (dynorphin A) and its receptor, respectively. This observation may consist with the pruritogenic action of mu opioid agonist and suggests the importance of the balance of mu and kappa opioid systems. A novel kappa opioid agonist TRK-820 expressed remarkable antipruritic action against mouse scratching induced by substance P or morphine. The drug also exerted antipruritic action in antihistamine-resistance uremic pruritus patients. These results propose a new hypothesis that mu opioid system is itch-inducible and kappa opioid system is itch-suppressive. These results also show that kappa opioid agonist is potential antipruritic drug to treat various pruritic diseases.

0360

Preventive effects of cystine derivatives on glycolic acid-induced cutaneous irritation

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Recently, glycolic acid has proved to be a versatile peeling agent and now widely used to treat acne, although it may induce skin irritation, characterized by stinging, burning and itching. Therefore substances capable of counteracting sensory irritation are of great practical interest. In an attempt to discover new anti-irritant compounds and the methodology of evaluating the skin irritation, we evaluated the efficacy of cystine derivative (N, N'- Diacetyl-L-cystine dimethyl ester; DACDM) in reducing chemically-induced skin irritation on the basis of own previous study. We examined as follows on the basis of double blind test utilized glycerin solution as control ; 1: Sample was applied to the cheek on the half face. 2: After 4 minutes, 20% glycolic acid was applied to the same spot. 3: The magnitude of the irritative sensation was evaluated each minute for the first 10 minutes after topical application using a visual analogue scale. We analyzed the experiment data by a type III analysis of variance assuming the test substance, the order of application and the individuality of subjects as the factors to influence the irritancy. The analysis revealed that DACDM reduced the irritative sensation by 12 percent. The response in the first application of glycolic acid on the cheek produced a response 10% greater than the one in the second. This study demonstrated that DACDM application as a pretreatment may act as a topical anti-irritant agent, especially, in reducing chemically-induced skin irritation and that the adopted methodology is effective for evaluating the efficacy of chemicals on the skin irritation. We propose that it is useful for the analysis of preventive effects of cutaneous irritation to utilize both of a visual analogue scale and analysis of variance.

0361

Topical mycophenolate mofetil 2% ointment therapy for psoriasis

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Mycophenolate mofetil (MMF) is a selective inhibitor of activated lymphocytes. It has been shown to have anti-psoriatic activities in previous non-controlled studies. However, its topical usefulness in treating psoriasis has not been systemically investigated. The purpose of this clinical trial is to evaluate the efficacy and side effects of topical MMF 2% ointment for stable plaque-type psoriasis. This is a randomized double blind placebo controlled pilot study. Five patients with stable plaque-type psoriasis were recruited with informed consent. Two comparable targeted lesions covering 0.5% to 2% body surface area from each patient were selected. One target lesion was randomly assigned to receive a 2% MMF hydrophilic ointment twice daily for 4 weeks followed by an optional 4 additional weeks. The other received the vehicle ointment. A pharmacist coded the MMF and the placebo ointments so that both the patients and the study physicians were blinded of the contents. Modified psoriasis area and severity index (PASI) was assessed for each lesion at week 0, 1, 2, 4 weeks (and optionally at week 8). One patient withdrew because of pruritus that developed on both lesions after two days into the trial. The remaining four patients finished the study. At week 4, the modified PASI of MMF-treated lesion decreased by 28% whereas the vehicle-treated lesion decreased by 15% ($p>0.05$). No significant side effects were observed. Contrary to previous non-controlled reports, topical MMF 2% ointment showed no significant efficacy for plaque-type psoriasis in this controlled trial.

0362

Use of bone marrow-derived cells for the treatment of recalcitrant chronic wounds

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Chronic wounds show a number of phenotypic changes that are believed to be responsible for the inability of these wounds to heal. Among the changes reported is evidence of senescence in cells derived from chronic wounds. These findings indicate that chronic wounds may be deficient in resident stem cells. Replacement of stem or progenitor cells might then correct some of these abnormalities. Recent evidence suggests that bone marrow can provide stem cells to non-hematopoietic tissues including the liver, heart and skin. With this interest, we examined the use of autologous bone marrow cells in the treatment of four patients with chronic wounds of greater than one year duration who had not responded previously to standard and advanced modalities of treatment including application of bioengineered skin and autologous skin grafting. All patients showed complete and sustained wound closure following treatment with autologous bone marrow derived cells. Histological analysis of the treated wounds strongly suggested engraftment of bone marrow cells into the wound bed. There was clinical and histological evidence of reduced scarring, dermal rebuilding and increased vascularity in the treated wounds. These findings provide initial proof of principle for the feasibility of using autologous bone marrow cells in diseased human skin, and suggest a promising approach to non-healing chronic wounds.

0363

Unusual CD4- CD8- malignant T cells expressing CCR4 Th2 chemokine receptor but bearing cytotoxic molecules in an adult T cell leukemia patient presenting with a purupuric eruption

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A high incidence of occurrence of skin eruptions is well known in adult T cell leukemia (ATL) and they include papules, plaques, nodules/tumors, and erythroderma. Tumor cells of ATL are CD4+ CD25+ T cells in most cases and have various cytokine expression/production patterns. We found that unusual double-negative malignant T cells were present in an ATL patient with a purpuric eruption. A 78-year-old Japanese woman had purpuric and erythematous lesions on the face and trunk. Approximately 60% of peripheral blood lymphocytes were positive for CD2, CD3, CD25, CD45RO, HLA-DR and TCR-alpha/beta, but negative for CD4, CD7, CD8, CD30, CD45RA, CD56 and TCR-gamma/delta. Lymphocytes with the same phenotype infiltrated in the skin lesions with lymphocytic vasculitis and resultant extravasation of erythrocytes. Southern blot analyses exhibited monoclonal rearrangement of TCR-c-beta-1 and biclonal integration of HTLV-1 proviral DNA in a skin biopsy specimen as well as circulating lymphocytes. The circulating tumor cells expressed Th2 chemokine CCR4 but not Th1 receptor CXCR3. An intracytoplasmic cytokine staining study revealed that the tumor cell did not produce either IFN-gamma or IL-4 upon stimulation with Ca ionophore and phorbol myristate acetate. Despite their Th2 chemokine receptor expression, the malignant T cells bore granzyme B as assessed by both intracytoplasmic flow cytometry and immunohistochemistry of biopsied lesional skin. These findings demonstrated that there exist ATL malignant T cells expressing Th2 type chemokine receptor but bearing cytotoxic molecules, which may lead to the purpuric erution in our case.

0364

Involvement of the endoplasmic reticulum stress in pathogenesis of Darier's disease

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The endoplasmic reticulum (ER) provides the site for folding and processing newly synthesized secretor and membrane proteins, and contributes to cellular Ca^{2+} signaling and storage. Various adverse conditions, collectively called ER stress, including a decrease of Ca^{2+} concentration in ER can interfere with ER function resulted in inducing accumulation of unfolded or misfolded proteins in ER. And these are collectively called ER stress. ER stress was known to upregulate expression of ER chaperones, inhibition of genetic translation, and inducing transcription factor C/EBP homology protein (CHOP) that leads to cell apoptosis. The cause of Darier's disease (DD) was identified to be the result of mutations in the *ATP2A2* gene encoding sarco/endoplasmic reticulum Ca^{2+} pomp (SERCA2) that actively transported Ca^{2+} from the cytosol into the ER lumen. In this study, we exposed DD culture keratinocytes to the conditions eliciting ER stress, and examined expression of the several ER chaperones which controlled the maintenance of protein tertiary structure and diminishing the load of detrimental ER misfolded protein. The results showed that the chaperones expression was increased in DD keratinocytes compared with normal. ER stress also induces CHOP/GADD153, a C/EBP family transcription factor, which is involved in ER stress-induced cell apoptosis. CHOP expression and cell death ratio by ER stree was more prominent in DD keratinocytes compared with normal keratinocytes. Our results indicate that DD keratinocytes are more prone to induce ER stress and suggested that ER stress may be involved in the pathogenesis of DD.

0365

Clinical evaluation of a cream containing MMP and PKC inhibitors on sensitive and irritated skin

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Matrix metalloproteases (MMPs) and PKC are implicated in several skin pathologies, especially inflammatory cutaneous disorders. More over, several molecules regulate the migration of skin dendritic cells, which are potent antigen presenting cells implicated in the primary T-cell-mediated immune response of the skin, among which MMPs and PKC. The aim of this study was to evaluate the clinical efficiency of an innovative topical product containing both MMPs and PKC inhibitors on sensitive and irritated skin predispose to allergy. 103 volunteers (mean age 39.7 years) were enrolled by 20 dermatologists. Among the panellists, 50 had a sensitive skin and 53 an irritated skin. The panellists have applied the product twice a day during 1 month. Subjective and objective criteria were evaluated by the dermatologist at T0 and T30. An auto-evaluation was performed by the volunteers. Its tolerance was also evaluated. The results showed a clear improvement of the objective and subjective clinical features, respectively -69 and -82%, of sensitive and irritated skin (erythema, dryness, desquamation, pruritus, stinging, burning). The auto-evaluation by the panellists showed that the cream has an immediate and long lasting effect (protective and apaising), and that it was able to improve the skin tolerance (66%). Moreover, the product was well tolerated and the cosmetic qualities were also delinated. In conclusion, this innovative cream containing an MMPs and a PKC inhibitor has proven its efficiency in the management of sensitive and irritated skin.

0366

Melanoma or not? Cancer testis antigens (CTAs) may help!

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CTAs are expressed in a variety of malignant tumors. No CTA expression was found in normal adult tissues, except male germ cells and placenta. To date, more than 20 CTAs or antigen families have been identified. Due to their tumor-associated expression, CTA expression may be useful for the distinction between benign and malignant neoplasms. However, the incidence of CTA expression in malignant tumors varies greatly and is often low. The present study was done to analyze the value of CTAs for the discrimination of cutaneous melanoma and nevus. Materials and Methods: 38 primary melanomas and 19 nevi (including 3 Spitz and 10 dysplastic nevi) were analyzed for their expression of CTAs by immunohistochemistry (IHC) using the following monoclonal antibodies (mAb)/to the following antigens: mAb MA454/MageA1; mAb 57B/Mage-A4, mAb ES121/NY-ESO-1. In a subset (n=5) of high risk melanomas, the CTA panel was extended to 3 additional CTAs: mAb CT7-33/Mage-C1, mAb M3H67/Mage-A3, mAbGage/Gage. Results: All 19 nevi were negative for MA454, 57B and ES-121. In melanoma, the immunoreactivity was as follows: MA454: 8/38 (21%), 57B 11/38 (29%), ES121 9/38 (24%). The expression pattern varied from focal to homogeneous. These data are lower than the results of a previous study, but are most probably due to the high number of low risk melanomas (<1mm tumor thickness, n=21) tested in our series. However, 19/38 (50%) were positive for at least one CTA and all 5 high risk melanomas (with 6 CTAs being tested) were positive for at least one CTA. Conclusion: Our analysis confirms previous studies, that CTA expression is present in cutaneous melanoma but not in nevi. Since the histopathological diagnosis of melanoma can be difficult in cases without clear-cut morphological signs of malignancy, CTAs may be useful in the determination of suspected malignancy in melanomas. The low incidence of paticular CTAs can be overcome by increasing the number of CTAs analyzed. Further studies are required to establish the optimal combination of CTAs for analysis of malignancy-suspect melanocytic neoplasms.

0367

Bimosiamose, a pan-selectin antagonist improves disease manifestation both in human psoriatic skin SCID mice and psoriatic patients--selectin antagonists as a new treatment strategy for inflammatory diseases

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The selectin family of vascular cell adhesion molecules is comprised of structurally related carbohydrate binding proteins, which mediate the initial rolling or "tethering" of leukocytes on the vascular endothelium following inflammation or injury. In T-cell-mediated diseases this process is one of the crucial events in initiating and maintaining inflammation. Therefore selectins are an attractive target for the development of new anti-inflammatory therapeutics. We investigated bimosiamose, a synthetic pan-selectin antagonist in psoriasis. Using the human psoriatic skin-SCID mouse transplant model, bimosiamose caused a decrease of disease severity as well as prevention of psoriasis in symptomless psoriatic skin. Therefore in a phase-II open clinical trial, we administered bimosiamose (600mg/day) over 14 days in five psoriatic patients. As demonstrated by assessment of psoriasis area and severity index (PASI), patients demonstrated significant improvement at the end of the study (p=0.02) which was paralleled by a reduction of epidermal thickness, a decrease of CD3+ and CD4+ cells and a reduction of P-selectin expression in immunohisto-chemical analysis. Flow cytometric analyses showed a reduction of peripheral CD45RO+ T-cells. Assessment of safety parameters showed no abnormal findings during the time of therapy. These data suggest that selectin antagonists may be a promising strategy for the treatment of inflammatory diseases.

0368

Topical application of 17β-estradiol modulate extracellular matrix proteins in aged human skin *in vivo*

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It has been known that decreased level of estrogen in postmenopausal women is associated with skin wrinkling and loss of elasticity. However, the changes of major components of extracellular matrix protein (ECM), such as procollagen, tropoelastin and fibrillin, after topical 17β-estradiol application to aged human skin are unknown. The purpose of this study was to determine the effects of topically applied 17β-estradiol on the expression of type I procollagen, tropoelastin and fibrillin in human aged skin. 17β-estradiol (0.01%) and its vehicle (70% propylene glycol, 30% ethanol) were applied topically to the aged (70~80y, 4 females and 4 males) human buttock skin under occlusion for 2 weeks (3 times/week). The skin samples were obtained by punch biopsy. We demonstrated that epidermal thickness in aged human skin was significantly increased after topical 17β-estradiol application. The immunohistochemical stain of type I procollagen protein demonstrated increased expression of procollagen in the dermal fibroblasts and extracellularly along the dermoepidermal junction. By western blot analysis, we also demonstrated that 17 β-estradiol increased the expression of type 1 procollagen protein significantly in human skin in vivo and decreased MMP-1 protein expression significantly in four out of 8 volunteers. Using immunohistochemical staining, we demonstrated that tropoelastin and fibrillin protein were stained in association with elastic fibers, and topical application of 17 β-estradiol in aged skin increased the tropoelastin and fibrillin expression significantly. These data suggest that topical application of 17β-estradiol may improve cutaneous ECM alternations in aged human skin.

0369

Bullous pemphigoid antigen 1 and a 190kD protein in addition to desmoglein 1 as immunological targets in superficial pemphigus with a combined cell surface and basement membrane immune staining pattern

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Pemphigus foliaceus and pemphigus erythematosus (PE) are frequently considered generalized and localized variants of the same disease process (i.e. superficial pemphigus). Distinct from pemphigus foliaceus, PE displays immune depositions at the dermal-epidermal junction, which suggests additional immunopathological mechanisms. We present three patients with clinical and histopathologic signs of superficial pemphigus, who all exhibit an immunomorphology characteristic of PE. Complement depositions in a granular-linear fashion were consistently found at the dermal-epidermal junction besides in vivo bound and circulating anti-keratinocyte cell surface antibodies. Histopathology showed subcorneal acantholysis, and all sera contained anti-desmoglein 1 but not anti-desmoglein 3 antibodies detected by enzyme-linked immunosorbent assays (ELISA). Additional antibodies against a 230kD protein and against a 190kD protein co-migrating with bullous pemphigoid antigen 1 (BP230) and with periplakin, respectively were present in all patients' sera. Since two sera specifically reacted with BP230 by ELISA, the presence of BP230-specific antibodies could be associated with dermal-epidermal immune staining in these patients. In PE dermal-epidermal immune staining is generally attributed to the deposition of immune complexes, while the presence of BP230-specific antibodies has not been reported in this disease previously. Perhaps, these patients' unique antibody profile permits to discriminate between patients with superficial pemphigus that display additional dermal-epidermal immune staining from those with conventional pemphigus foliaceus on a molecular basis. Further studies will be required to substantiate the frequency of this occurrence and to unravel its pathogenic significance.

0370

Ethnic variability in the skin response induced by Ivory Soap: a non-invasive, high-resolution evaluation

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Data regarding the susceptibility of irritant contact dermatitis (ICD) as a function of race has been contradictory. This study was designed to non-invasively evaluate and characterize ethnical differences in acute ICD reactions induced by diluted Ivory dish-soap. In the first experiment, 10 subject volunteers (5 Caucasians, 5 African-Americans) were recruited and patch tested to different concentrations of Ivory soap solutions. Each site was exposed for 24 hours and clinical scoring was performed at 4, 24, and 48 hours to determine the clinical threshold. In the second experiment, 20 subject volunteers (10 Caucasian, 10 African American) were patch tested and evaluated 24 hours after patch removal by reflectance-mode confocal microscopy (RCM), Fluorescence Excitation Spectroscopy (FES) and TEWL. At 24 and 48 hours, the clinical thresholds for irritancy in the black group were significantly higher ($p<0.032$, $p<0.016$), with a mean clinical threshold of 10% Ivory soap solution in the white and 20% in the black group. The second experiment demonstrated significantly higher clinical thresholds for ICD in the black compared to the white group ($p< 0.0001$). RCM features of ICD including stratum corneum disruption, parakeratosis and spongiosis were more severe in white than black skin at the 10% and 20% sites but differences were not significant at the subthreshold site. The FES spectrum for both groups showed a broad band at 300 nm (emission at 340 nm). The band increased in intensity in the first day after the application of the irritant ($p<0.04$) and in all cases returned to baseline by day 7. The mean TEWL at the 10% and 20% sites was significantly higher in the white group. The results of our non-invasive evaluation indicates ethnic variability and suggests that black skin may indeed be more resistant to contact irritants than it has previously been reported.

0371

Determination of effect of sun care skin nutrients on sun sensitivity in healthy women: a placebo-controlled, double-blind study

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The effect of dietary supplementation with a new preparation containing carotenoids derived from natural tomato and palm fruit extracts on skin color and melanization levels was investigated in this double-blind, placebo-controlled study. Forty healthy women, aged 18-50 years with s kin type II, were randomly assigned to two treatment groups. One capsule Imedeen Tan Optimizer™ providing 19.5 mg palm fruit extract, 3.8 mg lycopene, 10 mg Vitamin E and 60 mg Vitamin C or a placebo capsule was taken daily over a 12 week period. The first four weeks of the study simulated a preparation phase. During the remainder of the study, skin color on un-irradiated buttock skin was measured with a Chromameter with subsequent calculation of the Individual Typological Angle (ITA). The ITA provides an assessment of the skins melanization level and color tone, and thereby an indirect assessment of the sensitivity to sunlight . Lower ITA values reflect increased melanization level and a darker skin tone. The ITA was lower in the group supplemented with Imedeen Tan Optimizer™ (active group) after 33 days of supplementation, suggesting that the preparation phase before sun exposure should be at least 4 weeks. The ITA decreased further in the active group over the following weeks, resulting in statistically lower ITA values in the active group compared to the placebo by day 46 (Mann-Whitney U test p=0.0326). In conclusion, supplementation with Imedeen Tan Optimizer™ leads to decreased sun sensitivity as evidenced by a decrease in ITA in normal, un-irradiated skin. This supports an increasing protection of the skin against erythema beginning after approximately 4 weeks of supplementation.

0372

Quantitative topographical characterisation of psoriasis

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Psoriasis varies in clinical presentation, disease severity, treatment response and relapse. This makes for an indistinct and heterogeneous population. The classification of psoriasis allows for stratification of patients, but little is known about the frequency of patterns observed, whether age and sex affect distribution, and what predictors there are for therapeutic response. We undertook digital photography of patients with psoriasis and mapped the number and size of plaques together with the presence of scale and inflammation at six body sites. Surface area involvement was estimated at each site. 152 patients (83 m, 69 f) referred for phototherapy were studied, median age of 40 years. Median total plaque count was 180 plaques per patient, with no significant difference between males and females, but was lower in patients over 40 years. Small (<1cm) plaques were the most common size of plaques, comprising 77% of all plaques assessed. Medium (1-5 cm) and large (>5cm) plaques were less common, at 19% and 4% of the total plaque count respectively. The frequency of plaque size did not vary at different body sites. Most patients had a mixed pattern of presentation with small, medium and large plaques. Isolated small plaque (guttate) psoriasis was most common on the trunk, comprising 33% of the total patient group. The legs were the most common site for psoriatic plaques in terms of absolute numbers of plaques and the estimated %area of involvement. This predilection for the legs was negated when normalised for body surface area. Scale was present in 77% of patients with large plaques, but only 27% of patients with small plaques, suggesting scale is not an independent marker of disease severity, as currently used in PASI scores. The presence of inflammatory change was not affected by plaque size, but was more common on the trunk. Psoriasis is regarded as a having a symmetrical distribution:left-right comparison confirms no significant differences between each side in terms of plaque size and number. We plan to look at topographical characteristics as a predictor of therapeutic outcome.

0373

Reduction of postherpetic neuralgia with the use of valacyclovir-gabapentin combination therapy for acute herpes zoster

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Recurrence of the varicella zoster virus, known as herpes zoster(HZ), occurs in approximately 20% of the population and is most common after the fifth decade of life. Pain from postherpetic neuralgia (PHN) is one of the most debilitating sequelae of HZ. It has been shown that after conventional antiviral treatment of acute HZ, 40% of patients with severe pain at onset and 30% of those with moderate pain at onset continued to have pain at six months. In this study we analyzed the impact of gabapentin as an additive to valacyclovir therapy on the reduction of incidence of PHN. Herpes zoster patients age 50 and older, within 72 hours of rash onset and experiencing moderate to severe pain were enrolled. Patients were treated with valacyclovir one gram three times daily for seven days as well as analgesic medications as needed. Concomitant therapy with open label gabapentin 300 mg daily was started on the initial visit and the dose was escalated weekly as tolerated by the patients. Treatment with gabapentin was continued for either a four or eight week period depending on the patients' pain level. Preliminary data analysis reveals that, after the combination therapy, only 10% of patients have pain at the six month follow-up visit. This represents a 70% reduction when compared to conventionally treated patients with moderate to severe pain at presentation, approximately 35 % of whom would be expected to still have pain at month six. For those patients who still had pain at month six in our study, the average pain was only 1.8 on a 0 to 10 pain scale. These results show promise for the prevention and/or reduction of the pain of PHN by using gabapentin along with antiviral medications in the treatment of acute herpes zoster.

0374

Graft-versus-host disease, as seen by reflectance confocal microscopy and correlation with the corresponding histology

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Acute graft-versus-host disease (GvHD) is a major cause of morbidity and mortality after allogeinc bone marrow transplantation. Of all organ dysfunction associated with GvHD, the skin serves as the earliest and most frequent target. The current gold standard, skin biopsies, has major limitations. Reflectance confocal microscopy (RCM) is a novel technique that can visualize skin non-invasively and in vivo with high resolution and depth and offers significant advantages over routine biopsies. The objective of this study was to detect early signs of cutaneous GvHD before clinically apparent using RCM. RCM was performed in a well-established swine model of mixed chimerism at various time points after the allogenic bone marrow transplant. The imaged areas were subsequently biopsied for histologic correlation. Morphologic features as seen by RCM correlated well with the histology. Characteristics of (i)normal skin, (ii)early phase of dermal and epidermal inflammation without clinical evidence of skin rash, and (iii)skin necrosis followed by (iv)complete re-epithelialization and development of chronic GvHD were clearly demonstrated by RCM. Non-invasive in vivo RCM maybe the ideal tool for monitoring the early signs of cutaneous GvHD. It may allow the early detection of skin inflammation before it is clinically apparent. Prompter treatment may thus improve the outcomes of acute cutaneous GvHD.

0375

Estimation of the minimal tumor load detected by the serological melanoma markers S100B and MIA

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Melanoma cells express S100B and Melanoma inhibitory activity (MIA). Both of them can be detected in sera of melanoma patients and have been proposed as melanoma markers. In the present study, we made an effort to estimate the minimal tumor load detectable by the use of either marker. To this end, serum samples of 334 melanoma and 224 control patients were tested for their S100B and MIA concentrations. Results obtained were compared to the S100B- and MIA production rates of 15 early passage human melanoma lines. We found that the mean S100B/MIA concentrations and the prevalence of elevated S100B/MIA levels correlate with the stage of the disease. In the group of patients with stage IV disease, 84% were found to test positive for S100B and 76% for MIA. Evaluation of the melanoma lines' S100B- and MIA production rates revealed that 12 (80%) produced S100B which ranged from 282 to 16,680 µg/10e6 cells /72h. Eleven (73%) were shown to secrete significant amounts of MIA ranging from 12.9 to 2311.4 ng/10e6 cells /72 h. S100B was primarily released from dying cells while MIA was found to be secreted actively. Provided that the S100B- and MIA expression of the melanoma lines is representative of the patient's melanoma cells both in terms of expression level and pattern, the minimal tumor load detectable by S100B and MIA was estimated to range from 1.01x10e7 to 2.5x10e10 and from 6.7x10e8 to 2.5x10e10 cells, respectively. We conclude that melanoma cells of various patients differ with regard to their S100B and MIA production rates and propose that the latter represent one factor determining a given patient's serum S100B and MIA level.

0376

Wrinkles effacement by topical tretinoin is associated with an increase in the thickness of papillary dermal repair zone in human skin *in vivo*

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Topical tretinoin (RA) is an effective therapy of photodaged skin. Wrinkle effacement by RA is associated with an induction of procollagen I. Since procollagen requires further processing, its expression levels do not necessarily correlate with formation of mature collagen. In a mouse model of photoaging, topical RA increases the thickness of the dermal repair zone (DRZ), which represents formation of new collagen matrix. In human studies, however, such data are not available, perhaps due to longer treatment period required for its detection. Thus, we investigated the DRZ thickness following a long-term (>6 mths) use of RA in photoaging. Twenty-eight Caucasian females with moderate to severe photoaging were treated daily with 0.05% RA (Renova). Their mean age was 62 and mean therapy duration was 2.3 years (0.5-4.3). Fine wrinkles, coarse wrinkles and overall severity of photodamage were clinically assessed using a semi-quantitative grading scale [0 (none) to 9 (most severe)]. Crow's feet area was biopsied at baseline and end of treatment. The specimens were processed for Verhoeff-van Gieson elastic tissue stain and the DRZ thickness above solar elastosis but below the basal lamina was measured using image analysis software. Following RA therapy, fine wrinkles decreased from a mean of 7.1 to 5.0 ($p<0.0001$), coarse wrinkles from 7.1 to 6.1 ($p<0.0004$), and overall severity from 7.2 to 6.0 ($p<0.0001$). Histologically, papillary dermal thickness increased significantly from a mean of 17.5 µm at baseline to 40.3 µm ($p<0.00004$) after treatment. Correlation between clinical improvement and dermal thickening however, was not statistically significant. The thickened DRZ was identified to be collagen with Masson-trichrome stain. In conclusion, topical RA effectively treats photoaged skin. With its long-term use, there is a significant increase in collagen matrix deposition in the DRZ. This increased collagen matrix most likely is one cause of wrinkle effacement by RA.

0377

CTLA-4 expression in peripheral monocytes from patients with cutaneous T cell lymphoma

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Cutaneous T cell lymphoma (CTCL) is a malignancy of memory, effector, CD4 T cells that preferentially home to the skin. The pathogenesis of this chronic cutaneous malignancy however remains an enigma and no genetic aberration has been identified consistently that is diagnostic. To study whether potential abnormalities exist in peripheral blood monocytes (PBMC) from patients with CTCL, we have focused on the regulation of CTLA-4 expression, a gene that we have identified to be increased in CD45RO, CD4 T cells. This is a gene whose function at the biochemical level down regulates T cell activation. This product has previously been identified to be expressed on CD8 T cells and regulatory T cells. We hypothesized that this gene may be expressed at increased levels in peripheral blood cells in patients with CTCL since the malignant cell is a memory CD4 T cell. In analysis of resting peripheral T cells from normal volunteers, CTLA-4 is minimally expressed, less than 3% of cells as measured by flow cytometry. Upon induction by PMA, low level CTLA-4 can be rapidly induced in the peripheral T cells from normals, between 5-29%. In unstimulated PBMC from patients with CTCL, there was minimal expression of CTLA-4. However when CTCL cells were stimulated, we observed an increased ability to express CTLA-4 that correlated with more severe disease. Patients with stage 1 or II (n=10) expressed CTLA-4 in a pattern not statitically different from normals. However, patients with erythrodermic MF and those with high Sezary cells had 50-70% of their T cells positive for CTLA-4 staining when stimulated with phorbol esters. The molecular basis for this finding may reflect underlying biochemical abnrmalities in CTCL. These findings indicate that better characterization of CTLA-4 regulation may lead to further understanding of the pathogenesis of CTCL.

0378

CTCL patients have decreased T cell receptor rearrangement excision circles

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For many years, it has been appreciated that advanced stage CTCL patients are severely immunosuppressed. We previously reported that the T cell repertoire in these patients is dramatically contracted, despite the presence of often relatively normal absolute numbers of T cells. In this report, we investigate the hypothesis that the remaining T cells have expanded abnormally to fill this empty T cell "space". T-cell receptor rearrangement excision circles (TRECs) in CD3+ T cells are formed during gene rearrangement in the thymus and diluted by subsequent cell division. Their concentration is therefore a measure of unexpanded naive T cells relative to T cells which have undergone expansion. 25 patients with CTCL and 3 normal controls were recruited, and TREC concentration was determined using real-time quantitative PCR. Our results demonstrate decreased TRECs in many stage III/ IV patients and some stage I/ II patients, compared with both normal controls and previously published normal values. Subgroup analysis did not demonstrate a statistically significant correlation between TRECs and age, stage, or number of contracted profiles on spectratype analysis. However, among CTCL patients with a dominant peripheral blood T cell clone as determined by FACS analysis, there was a significant decrease in TRECs. Since a malignant CTCL clone is TRECs negative, the decrease in TRECs we observed suggests antigen independent expansion in the remaining pool of nonmalignant T cells. This decrease was not simply a dilutional artifact as demonstrated by experiments using Jurkat T cell clones mixed with normal T cells. We conclude that these data are consistent with antigen independent proliferation of T cells to compensate for loss of T cell diversity in CTCL.

0379

The pharmacokinetics and pharmacodynamics of efalizumab following 12 weeks of subcutaneous treatment in subjects with moderate to severe plaque psoriasis in a phase I, open-label, multi-center study

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Efalizumab is a recombinant humanized monoclonal antibody against CD11a, the alpha sub-unit of LFA-1, which is expressed on the surface of leucocytes. Binding of CD11a by efalizumab results in saturation of available CD11a binding sites (ABS) and down-modulation of cell surface CD11a expression (CSE). This event is believed to inhibit the activation of lymphocytes, therefore decreasing T-cell migration to the dermis and epidermis. The pharmacokinetics (PK) and pharmacodynamics (PD) of efalizumab were assessed for a total of 24 wks, including 12 wks of SC treatment with 1.0 or 2.0 mg/kg/wk. Steady-state serum efalizumab levels were achieved by 4 and 8 wks following administration of 1.0 and 2.0 mg/kg/wk efalizumab, respectively. At steady-state, peak serum concentration (Cmax) was 12 and 31 mg/mL, occurring ~2 days after a SC dose. Serum trough levels of efalizumab were 9 and 24 mg/mL and SC clearance (CL/Fss) was 24 and 16 mL/kg/day for the 1.0 and 2.0 mg/kg/wk doses, respectively. Following the last dose, serum efalizumab fell below the limit of quantification by 25 (1.0 mg/kg/wk) and 44 days (2.0 mg/kg/wk). During treatment, maximal saturation of ABS (>95% of baseline) and down-modulation of CSE (15-30% of baseline) on T lymphocytes was observed for both dose groups. Similar changes were also observed on B cells, NK cells, monocytes and neutrophils, but to a lesser extent. Absolute counts of circulating lymphocytes more than doubled compared to baseline while levels of monocytes and neutrophils remained stable. PD effects were reversible for both dose groups and by 5-8 weeks after the last dose, ABS and CSE returned to or exceeded baseline and lymphocyte counts were within normal range.

0380

Fibroblasts cultured from chronic wounds show decreased expression of TGF-beta type II receptor and altered Smad3 and p42/44 MAPK signaling

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The pathogenic mechanims underlying impaired healing in chronic wounds are largely unknown but, recently, increasing evidence points to phenotypic alterations in the resident wound cells. Cellular senescence and decreased responsiveness to growth factors, such as TGF-beta 1, are some of the reported abnormalities. Here we determined some of the possible mechanisms underlying these phenotypic changes in fibroblasts from chronic venous ulcers. We cultured fibroblasts from the edge of venous ulcers of nine patients and, in each case, compared the findings to fibroblasts cultured from acute wounds created simultaneously in the same patients. Using Northern analysis and affinity labeling, we show that venous ulcer fibroblasts have markedly (more than four-fold) decreased expression of the Type II receptor. This finding is not the result of genetic mutation, as shown by experiments with receptor satellite instability. The downregulation of Type II receptor expression seems to affect downstream signaling pathways. Thus, we found that venous ulcer fibroblasts have more than a 3-fold decrease in the phoshorylation of Smad3 and p42/44 MAPK in response to TGF-beta 1. Transduction of the venous ulcer fibroblasts with a constitutively phosphorylated form of the receptor abrogated these abnormalities. The proliferative and synthetic response of ulcer fibroblasts to TGF-beta 1 was markedly decreased. We conclude that venous ulcer fibroblasts have diminished expression of TGF-beta Type II receptor and that this, in turn, may lead to altered signal transduction abnormalities. Moreover, these findings suggest possible ways to address and treat the abnormal cellular phenotype in chronic wounds.

0381

An animal model for adult cutaneous mastocytosis

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The adult form of mastocytosis represents an abnormal growth and accumulation of mast cells in the skin and other organs. While at least two adult-onset mastocytosis mast cell lines exist (HMC-1 and RMT), at present there is no vivo model for the investigation of human cutaneous mastocytosis. Such a model might be particularly advantageous for the investigation of new treatments for this disease. Thus, the purpose of this study was to establish a murine model, so that the growth of human mastocytosis mast cells could be studied and the effect of therapeutic agents on these cells could be examined. Two established mastocytosis mast cell lines, HMC-1 and RMT which express autoactivated KIT, were used in this study. SCID Beige nude mice were injected intradermally on the flanks with different numbers (1, 5, 10, 50, 100 and 300 $x10^3$ cells in PBS) per site of HMC-1 and RMT cells. Human mast cell growth was detected in both cell lines within one week and by two weeks, mast cell tumors, ranging in size from 1x1 to 8x12 mm, were noted for both HMC-1 and RMT. Biopsies were obtained from the injection sites. For HMC-1, mast cell infiltration in the dermis was evident, but epidermotropism was not detected. The atypical morphology of these cells noted in culture did not change after being injected in the skin. New blood vessel formation, similar to telangiectasias seen in adult mastocytosis skin lesions, was noted in each injection site but was more prominent in the 300 and 100 X 10^3 sites. RMT cells also formed dermal and subcutaneous infiltrates. No epidermal involvement was seen, and in contrast to HMC-1, no neovascualrization was noted. We conclude from this study that HMC-1 and RMT mastocytosis cell lines can survive and grow in the skin of SCID animals. HMC-1 cells, but not RMT cells, appear to promote new blood vessel formation similar to changes seen in adult cutaneous mastocytosis suggesting the elaboration of endothelial growth factors. This animal model should prove useful for the investigation of human cutaneous mastocytosis and new therapies for this disorder.

0382

Hereditary hemochromatosis gene mutations associated with porphyria cutanea tarda

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Hereditary hemochromatosis represents the most common single gene mutation inherited in whites. An increased frequency of hereditary hemochromatosis gene mutations occurs in patients with porphyria cutanea tarda. Polymerase chain reaction analysis of peripheral blood for hemochromatosis gene (*HFE*) mutations is available for clinical use. We present 8 patients with porphyria cutanea tarda subsequently found to have hereditary hemochromatosis by hepatic pathology or harboring mutations in the *HFE* gene. Retrospective review of patients diagnosed with both porphyria cutanea tarda and hereditary hemochromatosis or *HFE* gene mutations between 1976 and 2000. Eight patients with porphyria cutanea tarda (6 male, 2 female; age range, 4-60 years; mean age at diagnosis of porphyria cutanea tarda, 42 years) were subsequently found to have hepatic iron overload or *HFE* gene mutations. Two patients had liver biopsy findings compatible with homozygous hereditary hemochromatosis. In the other 6 patients, *HFE* gene analysis revealed 3 homozygous C282Y, 1 compound heterozygous C282Y/H63D, and 2 heterozygous C282Y mutations. Seven patients (88%) had no specific signs or symptoms of hereditary hemochromatosis at diagnosis. Porphyria cutanea tarda can be an important cutaneous marker for patients with mutations of the *HFE* gene. *HFE* gene analysis should be performed in patients who present with porphyria cutanea tarda. The functional significance of C282Y and H63D *HFE* gene mutations, their frequency and association with porphyria cutanea tarda are reviewed.

0383

Clinical efficacy of a novel non-steroidal anti-inflammatory molecule in treating symptoms of rosacea: identification of inflammatory mediators inhibited by CX-452

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Chronic inflammatory skin conditions, including rosacea and eczema, are characterized by a release of pro-inflammatory mediators from multiple skin cell types leading to increased vascular permeability, release of proteases, and the emigration of immune cells into the tissue. Current treatments are dominated by corticosteroids that are only intermittently used because of associated side-effects. Development of novel topical non-steroidal anti-inflammatory molecules would provide relief without concomitant side effects. Screening of a phenolic chemical library identified CX-452, a naturally occurring compound that displays in vitro anti-inflammatory activity. To determine in vivo efficacy, topical formulations containing 1% CX-452 were tested by Franz cell analysis to optimize skin delivery. A formulation providing optimum flux of CX-452 into the skin was evaluated for efficacy in reducing the symptoms of rosacea. 30 patients with moderate rosacea were enrolled in a blinded, placebo-controlled clinical study. 20 patients were treated with 1% CX-452 lotion and 10 were treated with an identical lotion without CX-452. Patients were evaluated at 2 and 4 weeks using 4 indices: erythema, desquamation, uneven skin tone, and dermatitis, and an overall severity score recorded. Results showed that rosacea severity decreased significantly from baseline ($p<0.01$) in 13/18 subjects (72%) with an average improvement of 68%. In contrast, placebo-treated patients had no significant improvement. An additional clinical study for eczema found that 88% of patients (15/17) using CX-452 showed an average of 67% improvement in symptoms. Given the proven in vivo efficacy, topical products containing CX-452 may provide effective non-steroidal relief for a wide variety of inflammatory skin disorders.

0384

Advanced techniques to measure DMSO response of human skin

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Dimethyl Sulfoxide (DMSO) when applied to human skin results in a wheal and flare reaction. This has been used in the past to assess the barrier function of skin. The quantification of wheal and erythemal flare has traditionally been done clinically and by measuring hydration, transepidermal water loss, blood flow and ultrasonic imaging. The advancements in bioengineering technology have led us to study this phenomenon more thoroughly. We have observed the time-course of wheal evolution using a spectrophotometric technique. The start, maxima and the resolution of wheal were followed for 60 minutes. The surface structure and shape of the wheal were imaged using a fringe projection device. The optical coherence tomography showed cross-sectional pictures of the wheal including the swelling of the stratum corneum. Laser-Doppler blood flow imager documented the flare reaction. Changes in visco-elastic properties were observed using a ballistometer.

0385

A clinical model to evaluate pressure ulcer therapy

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Finding new safe and effective treatment modalities for the treatment of pressure ulcers represent a significant and complex problem for those working in the wound care area. Classical management calls for reduction of pressure and improved patient care. At present this arena is populated by numerous modalities. We present here the use of three models in which six safety and efficacy parameters can be globally assessed, for the selection of new modalities. These models provide useful preliminary evidence pertaining to the safety, efficacy and potential modes of action for this product and for others in development. Given the complex etiology of a pressure ulcer our system has to reflect both acute and chronic disruption of physiological processes. This research details studies addressing: peripheral blood flow; transepidermal water loss; reepithelialization, erythema and scabbing of laser created wounds; and the repeated insult patch test. The studies involved the use of an ointment whose active ingredients are Trypsin, Balsam of Peru and Castor Oil in a non-petrolatum formulation (Xenaderm®) which was compared to saline or untreated skin. In wounds created using an Erbium:YAG Laser (n=20), the ointment showed significant improvements in reepithelialization, erythema and scabbing compared to the control over the 10-day study. Laser Doppler evaluation (n=10) indicated that the ointment increased blood flow significantly compared to an untreated area at 1 and 3 hour post-treatment. A human repeated insult patch test (n=200) was conducted for 6 weeks (induction and challenge phases). No potential for contact irritation or allergy was seen with the ointment. The relevance of this methodology will be discussed and related to a basis for further studies to better evaluate therapies for complex wounds.

0386

The effect of bioengineered skin on cell proliferation

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Skin substitutes are increasingly being used in the treatment of difficult to heal wounds but their mechanisms of action remain uncertain. These constructs are able to synthesize and release growth factors and cytokines, and it is widely believed that this property is responsible for their stimulation of wound repair. In this in vitro study, we used a Boyden chamber co-culture system consisting of a neonatal fibroblast monolayer in the bottom well and various types of skin substitutes in the upper chamber. A filter with a pore size of 3 um separated the two chambers. Using histology, immunostaining, RT-PCR, and measurements of DNA synthesis in the fibroblast monolayer, we determined the proliferative response of the fibroblast monolayer when exposed to different types of skin substitutes: 1) a non-living construct (NLC) from pig intestinal mucosa; 2) a dermal living construct (DLC) consisting of neonatal fibroblasts in an absorbable matrix material; and 3) a living epidermal dermal construct (LEDC) consisting of living keratinocytes and fibroblasts in a collagen matrix. We found a 3-fold increase in DNA synthesis in the fibroblast monolayer incubated with NCL for 24 h but not with the other two constructs. Various sizes and amounts of the living constructs showed no difference in cell proliferation. Incubating the skin substitutes in DMEM prior to use in the co-culture system decreased the stimulatory effect of NLC on the fibroblast monolayer, but continued to have no effect with the living constructs. We conclude that the general assumption that living skin substitutes work primarily by the release of cytokines and growth factors needs to be re-visited. Other, more complex mechanisms may be at work.

0387

5% imiquimod cream for treatment of intraepithelial squamous cell carcinoma

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Cutaneous intraepithelial squamous cell carcinoma (Bowen's disease) is a common pre-malignancy, in which up to 5% of patients develop invasive squamous cell carcinoma. We present an 18-month prospective randomised placebo controlled trial of 5% imiquimod cream in the treatment of biopsy proven intraepithelial squamous cell carcinoma. 31 patients with a target lesion between 1-20sq.cm were recruited. Patients were excluded if they had psoriasis or were immunosuppressed. A random number generator was used to blind treatment allocation to either 5% imiquimod or matched placebo. Treatment was applied daily to the lesion, at night, for 16 weeks. Efficacy was assessed by biopsy, planimetry and photography at week 28, as well as by clinical assessment at week 52. Statistical analysis was undertaken with Chi square and Phi tests. The median age was 76 years (range 54 to 86). Lesions were on the lower limb (21), upper limb (8) and face (2). The median duration of lesions was 36 months and 11 had failed to respond to other treatments. The median surface area was 213 sq.mm and 2 lesions were ulcerated. 12 of the 15 cases receiving imiquimod and 14 of the 16 cases that recieved placebo completed the study. 7 cases treated with imiquimod and none of the placebo group had complete clinical and histological resolution, with no signs of recurrence at week 52. The response to imiquimod based on histology at week 28 was statistically significant, $p<0.01$ and $Phi<0.01$. Intention to treat analysis after the treatment period further validated these findings, the 2 placebo treated cases that withdrew had no clinical improvement, whilst the 2 cases on imiquimod that were withdrawn had clinical resolution. One case was lost to follow-up. 5% imiquimod cream appears to be an effective treatment for intraepithelial squamous cell carcinoma.

0388

Azelaic acid 15% gel versus metronidazole 0.75% gel as topical therapy for papulopustular facial rosacea: results of a 15-week randomized trial

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Given the chronic nature of facial rosacea, safe and cosmetically acceptable topical therapies that alleviate papules, pustules, and persistent erythema are needed. The efficacy, tolerability and safety of a new formulation of azelaic acid 15% gel (AzA gel) was compared with metronidazole 0.75% gel as treatment for moderate, papulopustular (stage 2) facial rosacea. In a 15-week, multicenter, double-blind study, 251 patients were randomized to twice-daily AzA gel or metronidazole gel. Efficacy variables included reduction in inflammatory lesions, erythema and telangiectasia ratings, investigator's global assessment (IGA) (static score), and investigator and patient overall improvement ratings. Efficacy analyses were carried out on the intent-to-treat (ITT) population using the last observation carried forward (LOCF) method. AzA gel demonstrated continuous improvement in inflammatory lesions and erythema through week 15, while the efficacy of metronidazole gel plateaued after week 8. At the end of study medication, AzA gel showed significantly greater reductions in nominal lesion count (-12.9) vs metronidazole (-10.7) (P=.0033) and a greater percent decrease in lesions (-72.7% vs -55.8%, respectively) (P=.0002). With AzA gel, erythema severity was "improved" in 56% of patients vs 42% with metronidazole gel (P=.017). No clinically relevant effect on telangiectasia was noted. AzA gel had a higher success rate based on the IGA (P=.0157), a higher overall improvement rating (P=.0049), and scored higher on the patient's overall assessment. Both treatments were cosmetically acceptability. No serious or systemic treatment-related adverse events were reported in either group. The most frequently reported AEs were mild to moderate facial signs and symptoms, with a higher incidence in the AzA gel group. Azelaic acid 15%, twice daily for 15 weeks, significantly and continuously improved two principal rosacea symptoms (inflammatory lesions and erythema) compared with metronidazole 0.75% gel.

0389

Proliferation and apoptosis during wound repair

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The edges of chronic wounds typically show acanthosis with the absence of epidermal migration, and there are questions about the migratory and proliferative activity of keratinocytes in chronic wounds. In order to determine the status and contribution of keratinocyte proliferation and apoptosis to the wound healing process, we performed immunohistochemistry for a proliferation marker (Ki-67) and apoptosis (TUNEL staining) on specimens obtained from the edges of acute wounds and chronic ulcers. Twelve patients with chronic venous leg ulcers underwent biopsies of normal thigh skin and chronic ulcers, and the initial thigh biopsy sites were re-biopsied after 48 hours to create an acute wound. The specimens were routinely processed for histology and immunohistochemistry. Immunostaining for Ki-67 was uniformly increased after wounding, with marked sparing of the advancing epidermal edge of acute wounds. However, Ki67 immunostaining was dramatically increased in ulcers. Apoptosis staining was very high at baseline in normal skin, and slightly increased after wounding. It was lower at baseline in chronic ulcers but increased at the advancing epidermal edge. With wounding, there was increased keratinocyte migration. The migrating keratinocytes closest to the edge of the wound, however, showed decreased proliferation as demonstrated by Ki-67 immunostaining. Contrary to what is commonly thought, chronic wound keratinocytes are actively proliferating. Keratinocyte apoptosis continues and in fact increases during healing, suggesting that it is part of the normal mechanism for wound repair.

0390

dHPLC based mutation detection for dystrophic epidermolysis bullosa

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Epidermolysis bullosa (EB) is a group of inherited skin disorders characterized by skin fragility after minor trauma. In its severest forms, EB may be lethal in the neonatal period, or severely debilitating, and therefore prenatal diagnosis is often requested by EB families, especially for the dystrophic (DEB) and junctional forms. To facilitate prenatal diagnosis, screening of the candidate genes specific to each of the major EB types has been undertaken in over 700 EB families by the DebRA Molecular Diagnostics Laboratory at Thomas Jefferson University in Philadelphia, PA. Among these families, 347 were DEB and 183 were junctional. Because of the relatively large size (32 kb) of the COL7A1 gene, the candidate gene for DEB, and the large number of exons (118) in it, direct sequencing is both labor intensive and costly. The method used for mutation screening until recently utilized heteroduplex scanning by conformation sensitive gel electrophoresis (CSGE), which resulted in the detection of 68% of all putative mutations in the COL7A1 gene in DEB patients and over 90% in JEB patients. Because of this low detection rate in DEB, a new mutation detection strategy was adopted utilizing denaturing high performance liquid chromatography (dHPLC, WAVE® Transgenomics). The mutation detection rate in the COL7A1 gene in DEB families using the dHPLC method approaches now 95%. The dHPLC method is less labor intensive than CSGE, is cheaper than sequencing the entire COL7A1 gene, can be automated for 96-well plate platform, and is more sensitive. The dHPLC method enhances the identification of COL7A1 mutations in DEB and results in more accurate prenatal diagnosis in families at risk for recurrence.

0391

Treatment with oral pimecrolimus significantly improves psoriasis with a clear dose-response effect

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There is a need for systemic therapies that are both effective and safe for treatment of moderate to severe psoriasis. Pimecrolimus is a non-steroid, selective inflammatory-cytokine inhibitor. It has been shown to display high cutaneous anti-inflammatory activity with low potential for affecting the systemic immune response. We performed a double blind, placebo controlled study to evaluate the efficacy and safety of oral pimecrolimus for the treatment of moderate to severe psoriasis. In a double-blind, placebo-controlled, parallel-group, dose-finding study, 143 adult patients with moderate to severe chronic plaque psoriasis were randomized to receive either placebo or oral pimecrolimus 10, 20 or 30 mg bid (1:1:1:1 assignment ratio). The study consisted of 12 weeks of double-blind treatment and a 12-week post-treatment follow up phase. Efficacy was assessed by: (i) Psoriasis Area Severity Index (PASI) and; (ii) the percentage of patients who were clear or almost clear of psoriasis as defined by the investigator's global assessment. The median change of PASI from baseline showed a dose-dependent effect and was greatest for the 30mg bid group. At 12 weeks, the median percentage reduction in PASI was 80%, 58%, 17% and 10% in the 30 mg-bid, 20 mg-bid, 10 mg-bid and placebo groups respectively. Following treatment with the highest dose of pimecrolimus, 43% of patients were clear or almost clear of disease following 12 weeks of treatment. In the two highest dose cohorts of pimecrolimus, more than half of the patients had not experienced disease relapse 10 weeks after stopping treatment. Oral pimecrolimus was well tolerated and no clinically significant changes were observed in physical or laboratory measurements. Oral pimecrolimus appears to be highly effective in the treatment of psoriasis and provides rapid and dose-dependent disease control. Based on its high level of efficacy and convenience, pimecrolimus has the potential to become a systemic treatment of choice for the management of moderate to severe psoriasis.

0392

Prolonged contact of bioengineered skin with the wound bed leads to increased closure rates of diabetic foot ulcers

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A bioengineered skin construct (BSC; Apligraf), consisting of human foreskin keratinocytes and fibroblasts in a collagen matrix, has been shown to accelerate the healing of diabetic neuropathic foot ulcers. In these and in other types of chronic wounds, the altered appearance of the construct after a few days in the wound bed can be confusing, and may erroneously lead to the suspicion of infection and be removed prematurely. There have been no studies to address the question of how long a bioengineered skin construct needs to remain in the wound for optimal results. To obtain information on this important question, we evaluated serial photographs of 74 diabetic foot ulcer patients who were treated with BSC. We examined the photographs at days 7 and 14, and assessed whether remnants of the bioengineered skin were debrided completely at these visits. We then determined whether the percentages of patients with healed ulcers by week 12 was affected by the removal of the construct. We found the following rates of complete wound closure: 46% for patients with BSC removed only at day 7; 47% for patients with BSC removed at both day 7 and day 14; 56% for patients with BSC removed only at day 14; and 72% for patients with BSC not removed at day 7 nor day 14. Together, the rate of complete wound closure for patients with BSC removed either by day 7 or 14 or both was 49% versus 71% for patients with BSC not removed by day 14 (p=0.047, two-sided chi-square test). In conclusion, maintaining BSC in the wound bed for at least 14 days leads to improved rates of wound closure.

0393

A 50% reduction in the Psoriasis Area and Severity Index (PASI 50) is a clinically significant endpoint

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A 75% reduction in the PASI score (PASI 75) is the current benchmark of primary endpoints for most clinical trials of psoriasis. Many consider this endpoint too stringent as it places effective therapies at risk of failing to demonstrate efficacy. We hypothesized that a 50% reduction in the PASI score (PASI 50) represents a meaningful change in a persons? life and thus is a better primary endpoint. To test this, we an analyzed PASI scores, quality of life (QoL) data, and desired re-treatment scores from a number of clinical trials in addition to the elements of PASI. This analysis shows: 1) The PASI score is not linearly reflective of psoriasis severity, eg a reduction in area of 95% without a change in redness, scaliness, and induration (ESI) can be only a 66% reduction in PASI; likewise a drop in ESI from an average of 3 to 1 (a very meaningful reduction) is not a 75% reduction. 2) Treatment with methotrexate, an effective psoriasis therapy, more frequently reaches PASI 50 than PASI 75 as evidenced by a recent open trial in which 63% of patients achieved PASI 50 vs. 26% achieving PASI 75. 3) Improvement in QoL exists at PASI 50, using the Dermatology Quality of Life Index a very significant improvement, (61% active vs. placebo) was found in several recent large clinical trials. 4) Patients achieving PASI 75 frequently defer therapy until they are well below PASI 50; a clinical trial where retreatment was patient initiated showed patients did not re-treat until their PASI dropped to an average of 20% improvement from baseline. 5) Effective, meaningful therapies are consistently differentiated from placebo at PASI 50 as evidenced by histologic and photographic parameters of clinical trials of alefacept, efalizumab, and etanercept. We conclude that PASI 50 equates to a clinically meaningful improvement in psoriasis and represents a discerning primary endpoint.

0394

Efficacy and tolerability of azelaic acid 15% gel as topical therapy for moderate, papulopustular rosacea established in two phase 3 randomized trials

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Rosacea is a common inflammatory facial dermatosis. The efficacy, safety, and tolerability of a novel aqueous gel formulation of azelaic acid 15% (AzA gel) as a topical therapy for moderate, papulopustular rosacea was evaluated. In two identically designed Phase 3, multicenter, double-blind studies, 664 patients were randomized to AzA gel or vehicle twice daily for 12 weeks. Efficacy variables included nominal and percent reduction in inflammatory lesions, erythema and telangiectasia ratings, investigator's global assessment (IGA) (static score), and investigator and patient overall improvement ratings. Efficacy analyses were carried out on data from the intent-to-treat (ITT population) using the last observation carried forward (LOCF) method. Pooled results of both studies are presented. With AzA gel, continuous reductions in inflammatory lesions and erythema were seen. At the end of study medication, AzA gel was superior to vehicle in mean reduction in lesion count (-9.9 vs -6.7, respectively) (P<.0001) and percent decrease in lesion count (-54.1 vs -39.1) (P<.0001). Significantly more AzA gel patients showed improvement of erythema than vehicle patients: 45% vs 28% (P<.0001). Therapeutic success on the IGA was achieved in significantly more AzA-gel patients vs vehicle: 61% vs 45% (P<.0001). AzA gel was rated superior on both physician and patient ratings of overall improvement. No notable improvement in telangiectasia was observed. No serious adverse events (AEs) were reported. Most treatment-related AEs were mild or moderate sensory cutaneous symptoms (eg, burning, stinging). Approximately 90% of patients rated both treatments cosmetically acceptable and local tolerability "good" or "acceptable despite minor irritation". These studies establish that azelaic acid 15% gel is a safe and tolerable topical therapy that provides continuous improvement of two principal symptoms of moderate, papulopustular rosacea (inflammatory lesions and erythema).

0395

Autoallergy—a novel pathomechanism in chronic urticaria

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Patients with chronic urticaria (CU) are reported to frequently exhibit IgG autoantibodies against thyroid antigens such as thyroid peroxidase (TPO) or thyroglobulin (TG). We, therefore, speculated that some CU patients also express IgE autoantibodies against TPO and/or TG and that CU in these patients may be autoallergic, i.e. due to skin mast cell activation by IgE-anti-TPO/TG and thyroid antigens. To test this hypothesis we have developed an ELISA assay, which allows for the quantification of IgE-anti-TPO serum levels. We found that 18% of 305 CU patients screened (but none of 34 healthy controls) exhibit IgE-anti-TPO of more than 7.5 IU/ml. One third of these CU patients showed IgE-anti-TPO levels of >20 IU/ml, i.e. >2-fold more than the highest level found in healthy controls. Interestingly, thyroidectomy in two IgE-anti-TPO+ CU patients resulted in a continuous drop of IgE-anti-TPO levels and complete remission of CU within 8 weeks after surgery, indicating that IgE-anti-TPO may be relevant for the induction of skin mast cell degranulation in CU. Furthermore, we found that preincubation of mast cells with IgE-anti-TPO and challenge with TPO results in strong mast cell degranulation and release of preformed mediators, supporting the view that IgE-anti-TPO-mediated mast cell activation may be a functionally relevant pathomechanism in CU. In summary, our findings encourage further investigations of autoallergic mechanisms in patients with urticaria diseases.

0396

Resistance towards arsenic trioxide involves constitutive activation of nuclear factor-kappa B in human cutaneous T cell lymphoma

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Resistance to apoptosis induction by chemotherapeutic agents is a major problem in cancer therapy. We recently showed that only high concentrations (about 5 micromolar) of arsenic trioxide (As2O3) induce apoptosis of human cutaneous T cell lymphoma (CTCL) cells in vitro. Since the Nuclear Factor-kappaB (NF-kB) family of transcription factors has been implicated in promoting survival of various hematopoietic neoplams, including mycosis fongoides, we investigated the status of NF-kB pathway in CTCL and its putative role in resistance to As2O3-induced apoptosis. Nuclear extracts from CTCL cell lines (HuT78, Seax, Myla) and tumoral cells from 8 patients with Sezary Syndrome (SS) were studied for NF-kB nuclear DNA-binding activity by electrophoretic mobility shift assay (EMSA). The localization of NF-kB proteins was analysed by immunocytochemistry and confocal microscopy with antibodies directed against the proteins p50, p52, p65, RelB and c-Rel. The expression of cytoplasmic NF-kB inhibitors (I-kB) and contra-apoptotic molecules of the Bcl-2 family was investigated by immunoblotting. Results demonstrate the presence of active NF-kB complexes (p50/p65) in the nucleus by confocal microscopy and ligation with kB elements by EMSA. Overexpression of Bcl-2 and BCl-Xl was detected in CTCL cell-lines and tumoral cells from SS patients, as compared to normal PBMC, whereas I-kB protein levels were not reduced. Constitutive nuclear translocation of NF-kB was almost totally reversed by cell treatment with the NF-kB inhibitors (i.e. SN50, ALLN, MG132), as shown by EMSA and cytochemistry. As2O3-induced apoptosis was potentiated by these inhibitors, as assessed by flow cytometric determination (annexin V / propidium iodide staining, mitochondrial transmembrane potential alterations) and immnoblot analysis of increased poly(ADP-ribose)polymerase cleavage. Our results demonstrate that constitutive activation of NF-kB contributes to the CTCL resistance to As2O3-induced apoptosis.

0397

Development of a new aqueous gel formulation of azelaic acid 15% as topical therapy for moderate, papulopustular rosacea

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Azelaic acid has recently been reformulated as a 15% gel (AzA gel) to maximize drug release, stability and cosmetic acceptability for improved patient satisfaction in the treatment of papulopustular rosacea. AzA gel is an opaque polyacrylic-acid-based hydrogel that is free of fragrances or alcohol. The high amount of wetting agent and low lipid content provide a cooling effect, ensure good skin humidification, and allow easy spreading. AzA gel has an adjusted pH of ~4.8 (close to the physiological pH of skin). AzA gel is nonsticky and has no whitening effect. The gel contains a higher amount of dissolved azelaic acid than the cream (25% vs 3%) as well as suspended microparticles (~1-10 μM). This probably contributes to a higher skin permeation and improved bioavailability. In a study with modified FRANZ cells and full-thickness skin of hairless mice, higher azelaic acid dose fraction (29%) was measured in the stratum corneum and viable skin with AzA gel compared with cream (4.2%) after a 24-hour dermal application of 14C-radiolabeled AzA 15% gel or 20% cream (dose: 8-16 mg/2 cm2). With regard to percutaneous absorption, after 8 weeks of AzA gel therapy, AzA plasma levels in 27 rosacea patients were well within the range associated with nutrition and endogenous metabolism. Azelaic acid has a very low toxicity, is nonteratogenic (Pregnancy Category B) and has been confirmed safe in worldwide clinical use. All AzA gel ingredients are Generally Recognized As Safe. In clinical studies in 457 patients treated with AzA gel, no serious treatment-related adverse events (AEs) and no incidence of phototoxicity, sensitization, or photosensitization were reported. The most frequently observed AEs were primarily mild to moderate and transient sensory symptoms (eg, burning, stinging, itching). The majority of patients rated local tolerability and cosmetic acceptability satisfactory or better. Thus, azelaic acid 15% gel combines improved stability and drug release, favorable cosmetic properties, and safety, making it a promising new topical therapy for facial rosacea.

0398

Srcasm in cutaneous disease

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Abnormal keratinocyte differentiation is associated with many cutaneous disorders, and can be identified histologically as an alteration in the ratio of compartments comprising the epidermis. Since it is known that Src-family tyrosine kinases (SFKs) play an important role in keratinocyte differentiation, it would be of interest to examine the levels of Srcasm: Src activating and signaling molecule, in cutaneous diseases. As Srcasm is a molecule that activates SFKs and links these kinases to signaling pathways regulating proliferation, differentiation, and apoptosis, cutaneous disorders in which these processes are abberrant may show altered Srcasm expression . In order to correlate Srcasm expression with cutaneous disease, mRNA in situ hybridization and immunohistochemistry for Srcasm were performed. Biopsies of common lesions with aberrant keratinocyte differentiation were examined including psoriasis, seborrheic keratoses, and squamous cell carcinoma. All psoriatic biopsies (n=9) demonstrated markedly increased levels of Srcasm mRNA throughout the lower epidermis relative to unremarkable skin and controls. Similarly, Srcasm mRNA was significantly increased within SCIS and basaloid cells of seborrheic keratoses. Interestingly, well-differentiated squamous cell carcinoma does not appear to contain significantly increased levels of Srcasm mRNA. Immunohistochemistry for Srcasm molecule in these lesions correlated with the levels of increased mRNA. Therefore, altered levels of Srcasm appeared associated with some cutaneous diseases, notably psoriasis

0399

The relationship of atopy, autoimmunity and age at onset in alopecia areata

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The lifetime risk of alopecia areata (AA) is approximately 1.4% and a positive family history is reported in 10-40% of cases. Several studies have now revealed association with genes of the MHC or chromosome 21 with possible immunological importance in the pathogenesis. Information concerning the distribution of age at onset of disease and the significance of atopy, autoimmunity and nail involvement is limited and we have examined these factors in a series of 450 cases of AA. The median age at onset in mild (patchy) AA was significantly later than in other clinical subtypes (29 vs 22 yr, $p<0.004$). Nail involvement was strongly associated with disease severity ($p<0.00001$). Associated autoimmune disease including thyroid disease, pernicious anaemia and vitiligo was much commoner in women with AA ($p<0.001$) but showed no clear association with disease severity. Atopy was commonest in patients with alopecia universalis (35%) and ophiasiform alopecia (50%) rather than in mild patchy disease (25%). The distribution of age at onset of AA in atopic individuals showed a unimodal pattern with peak incidence at 10-13 years of age in striking contrast to the non-atopic group that showed an additional peak at age 30-35. There was a trend towards a higher frequency of familial cases among female patients with AA, in keeping with some earlier series and a positive family history was less common in patients with AA of late onset. The observed patterns of heritability for alopecia areata are in keeping with a polygenic inheritance model and in future genetic studies, careful correlation of the clinical and genetic findings will be needed to understand the disease phenotpyes and guide the approach to developing new therapies.

0400

Results of two phase II multiple-dose trials of an anti-CD80 monoclonal antibody (IDEC-114) in patients with psoriasis

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IDEC-114 is an anti-CD80 (B7.1) monoclonal antibody developed using PRIMATIZED® antibody technology to minimize immunogenicity. In vitro and in vivo data demonstrated that IDEC-114 can effectively block antigen-specific immune responses and IgG production, and interfere with the progression of autoimmune diseases. Two Phase II, randomized, double-blind, placebo-controlled studies of IDEC-114 have recently been completed in patients with moderate to severe plaque psoriasis. Therapy consisted of IV infusions of IDEC-114 (1, 2.5, or 5 mg/kg weekly x 8; 10 mg/kg every other week x 8; 15 mg/kg monthly x 4) or placebo. A total of 228 patients were treated. A favorable safety profile was observed. The majority of adverse events (AEs) were Grade 1 or 2 and included headache, rigors, nausea, and upper respiratory tract infection. Two Grade 3 related AEs were documented: limb abscess (possibly related) and syncope (of unknown relationship). Both resolved. No anti-IDEC-114 antibody formation or T-cell depletion was detected. The mean Cmax and AUC were dose-proportional, with a median serum T1/2 of approx. 15 days. The primary efficacy endpoint was a 50% reduction from baseline in PASI score 2 weeks after completing treatment. Although clinical activity was reported in patients receiving IDEC-114, with greater responses in higher dose groups, no statistically significant difference between IDEC-114 and placebo was seen. In summary, multiple infusions with IDEC-114 were safe and well tolerated in patients with moderate to severe plaque psoriasis; however, the degree of clinical efficacy at these doses was limited. A Phase I/II study of IDEC-114 in relapsed or refractory, follicular NHL is ongoing. IDEC-114 is also being considered for other immunologic disorders.

0401

Poor prognosis of arthritis-associated pyoderma gangrenosum

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We have observed a refractory population of patients with pyoderma gangrenosum (PG) and tested the hypothesis that differences exist in healing in patients with arthritis associated PG (APG) compared to other groups of patients with PG. A retrospective chart review was performed of patients treated during a two-year period. The patients had noninfectious chronic ulcerations typical for PG, exclusion of relevant differential diagnoses and had consistent histopathology. Patients were treated with systemic anti-inflammatory and immunosuppressive therapy including prednisone, methotrexate, azathioprine, cyclophosphamide, or cyclosporine. Outcomes were compared between patients with PG and APG, including complete healing, percent change in wound size and duration of therapy. Percent decrease in wound size was defined as the change in wound size between the initial and final visit divided by size at initial visit x 100. The target ulcer was defined as the largest ulcer in patients with multiple ulcers. Paired t-tests and the sign rank test were performed. Eighteen patients, 10 patients with PG and 8 patients with APG, were studied. There were no significant differences between the two groups in terms of age, race, gender, comorbidity, treatments, and size of ulcer pretreatment. Seven of 10 PG ulcers healed vs. 2 of 8 APG ulcers. There was a greater percentage decrease in wound size in the PG vs. APG ulcers (mean decrease 78.9% vs. 23.4%; $p= 0.099$) and a shorter mean duration of treatment (8.7 months compared to 14.8 months). Ulcers of APG appear to be more refractory to treatment than ulcers of PG without arthritis, suggesting that APG ulcers represent a unique subset. It is possible that APG may be characterized by unique pathophysiologic mechanisms, or it may be that concomitant arthritis compromising venous return accounts for its refractory nature.

0402

Etanercept (ENBREL®) inhibits radiographic progression in patients with psoriatic arthritis

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Results from a 24-week study of etanercept in 205 patients with psoriatic arthritis (PsA) were previously presented (Meese, Arthritis Rheum 2001; 44:S90). Etanercept provided significant clinical benefit for both arthritis and psoriasis and was well tolerated. After all patients completed 24 weeks of blinded study drug and the database was locked, they could enroll into a 1-year open-label extension. A primary endpoint of the study was comparison of radiographic progression in patients treated with etanercept versus placebo. Radiographs of hands and wrists were taken at baseline and 24 weeks in the blinded study, at initiation of open-label treatment, at 1 year from original baseline, and at early termination during either part of the study. Pairs of independent readers, blinded to study treatment and chronological order of images, read digitized x-rays of hands and wrists and scored for erosions and joint space narrowing using a modified Sharp method (Sharp, Arthritis Rheum 1985; 28:1326-35). Additionally, other radiographic features of PsA were evaluated. X-rays were read and scored from all 205 patients in the study. There was significantly less radiographic progression in the etanercept group compared with the placebo group. The median rate of change from baseline to 1 year in total Sharp score was -0.03 units in the etanercept group versus +1.00 units in the placebo group ($p = 0.0001$). Median rates of change in erosion scores were -0.09 units/year in etanercept patients versus +0.66 units/year in placebo patients ($p < 0.0001$), and in joint space narrowing were +0.05 units per year in etanercept patients versus +0.34 units/year in placebo patients ($p = 0.0438$). Radiographic progression was measurable in this controlled study of patients with PsA. Patients receiving placebo had progressive joint destruction as shown by increases in erosions, joint space narrowing, and total Sharp scores. Structural damage was significantly inhibited in patients treated with etanercept.

0403

Cytokine response of atopic dermatitis patients to patch testing with dust mite and yeasts

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We determined mRNA cytokine expression on 8/14 atopic dermatitis (AD) patients (pts) following postive atopy patch tests (APT) to dust mite or yeast. Patch tests were applied to intact or scarified skin of the upper back with serial concentrations of *Malassezia sympodialis* (ms), *Candida albicans* (ca) and *Dermatophagoides pteronyssinus/farinae* (df). Application sites were examined at 1, 6, and 48-72 hours after patch application. Punch biopsies were obtained from positive APT, total RNA was extracted and RT-PCR was performed. 1/14 pts was APT positive to 1%, 10%, and 20% df at 1 hr. Biopsy from the 20% df site was positive for IL-12p40. At 6hr, an additional pt responded to df and was positive by PCR for IL-12p35 and IL-12 p40. Additionally, this pt was also APT positive to 80% ms on scarified skin, and IL-12p40 positive but IL-12p35 negative. Between 48-72hr, an additional 8 pts responded to either ca, ms or df; these responders tended to be those pts with a history of severe AD. Two pts declined biopsy and were not included in this analysis. Of the remaining 6, all were APT positive to df and one pt was also positive to ms. Three pts out of these 6 were positive for IL-12p40 only, two were positive for both IL-12p40 and IL-12p35, while one patient was negative for both. 1/8 of the pts who was APT positive to 20% df and 80% ms at 6hr was also positive for IL-8. All 8 pts were negative for IL-6 expression from APT positive biopsy sites. IFNγ was detected in biopsies taken at 48-72 hours in 4/8 pts. Cytokine analysis of biopsy sites from APT positive to df, ms and ca suggest that type I cytokines occurred within hrs of application of protein extract from commensal yeasts. Patients with AD may develop protein contact dermatitis to proteins such as those derived from commensal yeasts on the skin due to abnormal barrier function. Interestingly, approximately half the pts demonstrate a type I cytokine response (IL-12p40, IFNγ) early in evolution of their APT reaction.

0404

Topical delivery of vitamin C and magnesium ascorbyl phosphate enhanced by lasers and microdermabrasion

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The objective of this study was to evaluate the ability of lasers and microdermabrasion, the skin resurfacing techniques, to enhance and control the in vitro skin permeation and deposition of vitamin C. The topical delivery of magnesium ascorbyl phosphate (MAP), The prodrug of vitamin C, was also examined in this study. All the resurfacing techniques tested showed significant increase on the topical delivery of vitamin C across and/or into the skin. The Er:YAG laser showed the greatest enhancement on skin permeation of vitamin C among the techniques tested. The laser fluence and its spot size were found to play an important role in controlling the drug absorption. An excellent correlation was observed in the Er:YAG laser fluence and transepidermal water loss (TEWL), which is an estimation of skin disruption. The permeation of MAP could not be enhanced by the Er:YAG laser. The CO2 laser at the lower fluence could promote vitamin C permeation without any ablation of stratum corneum (SC) and epidermal layers. On the other hand, the CO2 laser at the higher fluences further enhanced drug permeation by the ablation effect. The microdermabrasion ablated the SC layers with the minimal disruption of skin according to TEWL levels. The flux and skin deposition of vitamin C across microderabrasion-treated skin was approximately 20-fold higher than that across intact skin. The techniques used in this study may be useful for basic and clinical investigation of enhancement of topical vitamin C delivery.

0405

Global safety and efficacy of more than 5 years of etanercept (ENBREL®) therapy in rheumatoid arthritis

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The safety and efficacy of etanercept in rheumatoid arthritis (RA) patients has been demonstrated in multiple clinical trials and from over 4 years of post-marketing experience. Observations from RA trials reflect experience with 2054 patients, some of whom have been receiving treatment for more than 5 years (5549 patient-years). All patients participating in RA trials of etanercept were eligible to enroll in open-label extension studies. Results from patients in all RA trials and open label extension studies both in North America and Europe are presented here. The safety and clinical efficacy of etanercept was evaluated in these patients at routine intervals. Results from 1442 patients (4044 patient-years) in North America and 612 patients (1505 patient-years) in Europe demonstrate continued safety of etanercept for more than 5 years of therapy. Overall, the rate of adverse events remains low. The frequency of infections requiring hospitalization or intravenous antibiotics was 0.04 per patient-year in the total population (5549 patient-years), which is comparable to the rate in control groups (0.04 per patient-year) in controlled trials. The incidence of malignancies was similar to the expected incidence calculated from the Surveillance, Epidemiology and End Results (SEER) database (49 observed vs. 48 predicted). Long-term efficacy has also been sustained and substantial. After 5 years of etanercept therapy (n = 119 in North America), 24% of patients had no tender joints, 24% had no swollen joints, and 16% had disability scores of zero (per Health Assessment Questionnaire). Patients treated with etanercept have been able to reduce or discontinue their corticosteroids while maintaining a clinical benefit. Etanercept continues to have an excellent safety profile for the treatment of rheumatoid arthritis for more than 5 years. Clinical efficacy is sustained for the duration of treatment.

0406

Prolonged response durability following ENBREL®(etanercept) monotherapy

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Etanercept was evaluated in a phase II multicenter, double blind randomized 24-week study, in which 56% of etanercept patients achieved 75% improvement in PASI (Gottlieb, et al., J Invest Dermatol 2002;119:234). In order to evaluate the durability of response to etanercept and safety following withdrawal, patients were studied further during an extended follow-up period. 112 psoriasis patients received etanercept 25 mg (n = 57) or placebo (n = 55) subcutaneously twice weekly in the 24-week portion of the study. 17 patients in the etanercept group that completed the 24-week study entered an extended study period and had follow-up evaluations of their psoriasis through week 52. Twelve of these 17 had achieved PASI 75 during the blinded study. The time to disease relapse was calculated from last dose of etanercept to date of return to 75% and to 50% of the baseline PASI. The start date of systemic therapy was used if that occurred prior to reaching the threshold PASI. The 17 patients in the etanercept group who entered the extended follow-up observation period had a median of 24.3 weeks before they reached at least 75% of their baseline PASI. The 12 patients who had achieved a PASI 75 at Week 24 had a median of 26.4 and 21.3 weeks following their last dose of study drug before they reached at least 75% or 50% of their baseline PASI, respectively. In all patients who were followed after etanercept discontinuation the disease relapse was gradual, and in no case did a patient's follow-up PASI score exceed the baseline value for that patient. No rebound flare, conversion to pustular psoriasis, or appearance of new skin manifestations was observed after discontinuation of etanercept. Etanercept monotherapy is efficacious and well tolerated in the treatment of psoriasis, with a response durability of over 5 months. Discontinuation of etanercept was not associated with rebound flare or other untoward events.

0407

A case of Netherton syndrome

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A 2-year-old male presented erythematous macules, bow-shaped scaly lesion and partly ichthyosiform skin on his trunk and extreemities a few months after birth. At delivery, no abnormality was found in his vital and appearance. His hair of scalp was found to be trichorrhexis invaginata, so called bamboo hair. Skin biopsy was performed and achantosis of epidermis and slight inflamation of dermis was found. Clinicaly and pathologicaly, Netherton syndrome was suspected. The patient's familly were researched and his cousin was found to have bamboo hair.

0408

***In situ* elastolytic activity in cutaneous melanoma**

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Cancer invasion is accompanied by a tissue remodeling process that involves a proteolysis of extracellular matrix, including elastin with further elastin-derived peptides (EDPs). In a series of biopsy specimens from 22 melanomas (including 6 cutaneous and 7 pulmonary metastases) and 5 nevi, elastic fibers were stained with orcein on histologic sections and morphologically and quantitatively analysed. Sections were studied by immunohistochemistry for the expression of the following matrix metalloproteases (MMP): MMP-2, MT1-MMP, MMP-1 and TIMP-2, by tumoral and stromal cells. Whatever Breslow index, analysis of orcein stained sections revealed that elastic fibers have almost disappeared, within tumor and peri-tumoral lymphocytic infiltrates, while they were more preserved within nevi. In primary melanomas, a degraded, thin appearance of elastic fibers (until total absence) was observed at the immediate vicinity of the tumour, but never in nevi. A preserved or "pushing" appearance, was less frequently observed in primary melanomas, but was common in nevi. None tumour-induced elastosis was observed. MMP-2, MT1-MMP and MMP-1 were strongly and similarly coexpressed by skin and pulmonary melanoma cells. A relationship with malignant phenotype was not evidenced, since their expression was also noted in the 5 nevi. These 3 proteases were significantly more expressed in melanomas with Breslow >0.45 mm. A weak expression of TIMP-2, in melanoma cells, was observed in only 2 cases. Overexpression of MMPs was not observed in peri-tumoral stromal cells compared to healthy skin. In conclusion, elastolytic activity appears to be greater in melanomas than in nevi, with particular presence of EDPs, suggesting acquired invasive capacity by tumoral cells. Since these peptides are able to stimulate MMP-2 production and activation, they likely represent, a way for overexpression and activation for elastases, with possibility to amplify tumoral response in melanomas.

0409

Efficacy and safety of ENBREL® (etanercept) in patients with psoriasis: results of a phase III study

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In psoriasis psoriasis and psoriatic arthritis studies, etanercept produced significant improvement in psoriatic skin disease. This phase III study was performed to confirm the efficacy and safety of etanercept monotherapy in patients with psoriasis. In this multicenter, blinded, randomized study, patients received either etanercept at one of 3 doses (25 mg once weekly [25QWk], 25 mg twice weekly [25BWk] and 50 mg twice weekly [50BWk]), or placebo, by SC injection for 12 weeks. After 12 weeks, in a double blinded manner, all placebo patients began etanercept at 25 mg SC twice weekly, and all patients in the three active groups continued on their initial dose, through 24 weeks. The primary endpoint was the proportion of patients achieving at least a 75% improvement in Psoriasis Area and Severity Index (PASI) at 12 weeks. 652 patients enrolled and were dosed in the study. Groups (n = 160, 162, 164 for etanercept 25QWk, 25BWk, and 50BWk, respectively; n = 166 for placebo) were well matched for patient age, psoriasis duration, and disease severity. The percent of patients achieving PASI 75 at the 12 week primary endpoint was significantly higher in the etanercept groups (25QWk, 25BWk, 50BWk) vs. the placebo group (14%, 34%, and 49% vs. 4%, respectively; p value =0.0006, <0.0001, and <0.0001, vs. placebo, respectively). In the three groups treated initially with etanercept, efficacy continued to improve, with 25%, 44%, and 59% of patients, respectively, achieving PASI 75 at 24 weeks. Statistically significant improvements in patient and physician global assessments and DLQI confirmed efficacy of etanercept therapy. Etanercept was well tolerated. Numbers of patients reporting adverse events were similar between etanercept and placebo groups at 12 weeks. Conclusion: Etanercept monotherapy was efficacious and well tolerated in the treatment of psoriasis.

0410

Neonatal head circumference, neonatal weight, and risk of eczema, hay fever, and asthma in a large cohort of adolescents from Sheffield, England

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Neonatal physical characteristics have been hypothesized to be markers of in utero thymic development. Previous studies have linked greater birth weight to subsequent development of eczema, and larger head circumference and lower birth weight to asthma. To investigate potential associations between neonatal head circumference and weight and atopic diseases in adolescents from Sheffield, England, we linked responses to a questionnaire inquiring about physician-diagnosed atopic disease to previously recorded neonatal measurements. Logistic regression methods were used to relate diagnoses to neonatal measurements and potential confounders. Of 10,809 subjects, 16.2% reported eczema, 16.5% hay fever, and 18.0% asthma. After adjusting for sex, age at time of the questionnaire, maternal age and gestational age at birth, number of older and younger siblings, time since birth of next older sibling, neonatal sickness, neonatal feeding type, and parental educational backgrounds, hay fever was the only disease associated with neonatal measurements. Comparing the highest to the lowest fifths of distributions, lifetime prevalence of hay fever was positively associated with neonatal head circumference (adjusted odds ratio 1.23, 95% CI 1.03-1.47) and with birth weight (1.17, 0.99-1.39). Hay fever was inversely related to the ratio of head circumference to birth weight (0.89, 0.75-1.05) and to gestational age. These associations were not substantially altered by further adjustment for gestational age. Greater neonatal head circumference may be associated with an increased risk of hay fever, but the inverse relationship between hay fever prevalence and the ratio of head circumference to birth weight challenges the prior hypothesis that greater head circumference relative to body mass reflects abnormal thymic development in utero, increasing the likelihood of allergic sensitization.

0411

The increased risk of skin cancer is persistent after discontinuation of PUVA: a cohort study

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Psoralen + ultraviolet A (PUVA)-treated psoriasis patients are at increased risk for squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). To assess the persistence of cancer risk among patients who have discontinued PUVA and the risk of a first tumor with the passage of time, we prospectively studied incidence and risk factors in a cohort of 1380 psoriasis patients treated with PUVA between 1975 and 2001. We observed a total of 27,840 person-years of which 16,537 (59.4%) person-years were considered years without PUVA exposure and documented 2,147 SCCs and 1,363 BCCs. In a multivariate analysis, which adjusted each risk estimate for all significant predictors of tumor risk, level of PUVA exposure was the single most strongly factor associated with SCC and BCC risk. Stopping PUVA for less than 15 years not significantly associated with a reduction of SCC risk. Fifteen years or more after discontinuation of PUVA the risk reduction was close to significant (IRR=0.79; 95%CI=0.62, 1.01). The risk of a first tumor is associated with level of PUVA exposure. After 25 years, about 7% of patients with ≤ 200 PUVA treatments and more than half of the patients with ≥ 400 treatments develop at least one SCC. Patients exposed to ≥200 treatments had a significant higher risk of a first BCC and after 25 years, almost one third of these patients developed at least one BCC. In conclusion, substantial exposure to PUVA dramatically increases the risk of non-melanoma skin cancer and prior exposure to PUVA remains an important issue in the management of patients because the cancer risk associated with PUVA is persistent.

0412

Is there a relationship between atopic dermatitis and non-melanoma skin cancer?

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Currently, we do not know if there is an association between atopic dermatitis (AD) and/or its therapies and non melanoma skin cancer(NMSC). Isolated reports have appeared in the past describing individuals with AD who have developed NMSC. While it is tempting to attribute these reports of NMSC to AD and prior therapies, there is limited information that associates those with AD to an increased occurrence of NMSC. The goal of this case-control study was to investigate whether individuals with a history of AD are more likely than other dermatologic patients to develop NMSC. Cases (patients with NMSC) and controls (patients with warts, seborrheic keratosis, acne rosacea, or dermatophyte infection and no history of NMSC) were randomly selected from an administrative record database of all outpatients seen in the Department of Dermatology of the University of Pennsylvania. Patients were contacted by mailed survey to determine the odds of an association between a history of AD, defined by the UK Working Party Diagnosis Criteria, and NMSC. 69.8% (N=3,207 out of 4,591) of those contacted completed the survey. 18.4% (254) of the controls had a history of AD and 13.7% (210) of the cases. Using logistic regression, the unadjusted odds ratio of having AD for those with a NMSC as compared to those with out a NMSC was 0.70(95% confidence interval 0.57-0.85). The fully adjusted odds ratio (confounders: age, gender, ethnicity, and topical steroid use) was 0.78(0.61, 0.98). Varying the definition of AD had little effect on our result. Therefore, it does not appear that patients with a history of AD are more prone to develop NMSC than other dermatology patients. As a result, future long-term studies of any new therapy must focus on the incidence of NMSC in AD patients and not assume that patients with AD have an increased risk of NMSC due to their disease or previous therapies.

0413

The impact of atopic dermatitis on young American children and their families: a qualitative analysis

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Atopic dermatitis greatly affects the quality of life of afflicted children and their families. The impact on psychological, social, and physical functioning is complex, and likely varies among children of different ages. The effects of atopic dermatitis on the quality of life of very young American children and their families are not well understood. To develop a conceptual model for understanding and improving the quality of life of these patients, in-depth, structured interviews were conducted with 26 parents of children with atopic dermatitis, ages 3-69 months, and 6 expert clinicians. Interviews were tape-recorded and transcribed. The specific ways in which atopic dermatitis affects the lives of children and their families were rank ordered and categorized into domains according to similarity in content; these domains were used to compose a conceptual framework. The respondents reported 181 different and specific quality-of-life effects, which were categorized into 8 domains: physical health, emotional health, physical functioning, and social functioning, for both the child and the family. The most common reported effects on children were sleep disruption, itch, increased crying, diet restriction, pain, and decreased social contact; the most common reported effects on the family were co-sleeping and sleep deprivation, unsolicited advice from relatives and friends, worry, staying home more, guilt, and sadness. These empirically-derived domains form the basis for a comprehensive conceptual framework for the ways in which atopic dermatitis affects the quality of life in young American children. Moreover, the framework can be used to develop a quantitative measure of this important health outcome for atopic dermatitis.

0414

The assessment of Fitzpatrick skin type using digital images

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The use of digital imaging for the determination of skin pigmentation has potential utility in large data collection studies such as the National Health and Nutrition Examination Survey (NHANES). As part of the effort to include skin pigmentation data collection in the future of NHANES, a study was undertaken to assess the validity of including a number of dermatological diagnoses, among them Fitzpatrick skin type using digitalized images. The primary objective of this portion of the study was to determine whether dermatologists could reliably determine skin type from digital images as compared to an in-person assessment. Secondary objectives included agreement between: 1) self-report of skin type and in-person exam, and 2) measurement of skin pigmentation using a Derma Spectrometer (Cortex Technology, Hadsung, Denmark) versus the in-person exam. 498 of 558 subjects who participated in a free skin cancer screening had photographs taken. The study included 151 men and 347 women, who classified themselves as follows: 418-white, 29-black, 12-Hispanic, and 35-other. Ten months after the screening, 2 dermatologists (D1&D2) read the images and recorded the skin type. Percent agreement and simple Kappa statistics (κ) were used for analyzing the dichotomized nominal categorical data, "burn" (types I&II) or "tan" (types III-VI). Agreement between self reported skin type and in-person exam was poor (κ=.301)and fair (κ®.4) for in-person examiner and D1&D2. The concordance for "burn" (types I&II) was ®50% for all but self reported compared with in-person reading. Using the in-person dichotomized skin type classification as criteria, the melanin index (dermatoscope reading) was statistically higher (p•.0001)for "tan" (index=35) than for "burn" (index=31.53). Thus, digital images can be used for determining skin type with acceptable reliability when dichotomized to groups that have a high risk of burning and those that have a lower risk of burning.

0415

The separation of year of birth from other factors determining the incidence and stage of disease at diagnosis of melanoma

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Birth cohort effects show that sun exposure in childhood and adolescence plays a major, but unquantified, role in the causation of malignant melanoma. To explore these cohort effects we have extended the poisson model traditionally used in the analysis of data from cancer registries. Birth cohort effects represent responses to risk factors within the patient - early damage, bad habits acquired early etc. - and not societial changes, such as more available or better care. The melanomas were registered with the population based SEER program in 1975-99. The two factor poisson model generates a constant for the whole model and factors for year of birth and year of age. Using these estimates and the population at risk, we predicted the expected numbers of cases in each cell for each stage of the disease. Then we removed the year of birth term from the prediction model and, with the other parameters unchanged, predicted new expected numbers of cases for each cell. The difference between the two sets of expected numbers represents the birth cohort effect. Cohort effects for cases diagnosed when metastatic are smaller than for locally invasive melanomas. In turn, cohort effects for locally invasive cases are smaller than for those cases diagnosed *in situ*. For cases diagnosed 1995-99, for males year of birth accounted for 98% of the *in situ*, 86% of the locally invasive, 73% of the regional and 57% of the cases with distant spread. For females the proportions were 97% *in situ*, 81% locally invasive, 63% regional and only 49% of the cases with distant spread at diagnosis. Melanomas associated with year of birth are different from other melanomas.

0416

Surveillance for colonization with methicillin-resistant *Staphylococcus aureus* in a dermatology outpatient clinic

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During the past twelve years there was an increase of prevalence for methicillin-resistant Staphylococcus aureus (MRSA) in German hospitals. There are only few reports discussing MRSA-infection in dermatology, and most of them focus on hospitalised patients and on MRSA spread, respectively. Only two reports exist on MRSA investigations in dermatology outpatient clinics. Therefore, a prospective study was performed on 231 dermatological outpatients, suffering from inflammatory skin diseases (n=90), from venous leg ulcers (n=58), and from basal cell carcinoma (BCC)(n=83). The following data were abstracted from the medical history: decubital ulcers, former antibiotic consumption, number of attendences to hospitals, surgery with general anaesthetic, diabetes, hemodialysis, infusion via catheter, physiotherapy. Anterior nares, perineal region and lesional skin was sampled in each patient. All swabs were collected using commercially available transportation media. Swabs were cultured on Columbia agar and mannitol salt agar using microbiology standard procedures. The available MRSA strains were further characterised by pulsed-field gel electrophoresis after digestion with the restriction enzyme SMaI. MRSA-positivity was detected in 1/32 patient with atopic eczema, 2/3 with gram-negative foot infection, 2/2 with vasculitis, 1/1 with myiasis, 1/1 with cheiropompholyx, 1/1 with a clavus, 1/1 with perianal eczema, 1/1 with vulvar eczema, 6/58 with venous leg ulcers, 0/83 with BCC. Statistical analysis revealed that the presence of decubital ulcers as well as the type of skin disease was significantly associated with MRSA-positivity. No mupirocin resistance could be detected. According to our results particularly patients with decubital ulcers and venous leg ulcers bear the risk of being colonised with MRSA and, therefore, should be screened.

0417

Allopurinol is the medication most frequently associated with Stevens-Johnson syndrome or toxic epidermal necrolysis in Europe

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The risk of allopurinol-induced SJS or TEN was investigated based on a European case control study (EuroSCAR study). 379 validated cases with SJS or TEN and 1505 controls were enrolled from April 1997 to December 2001. Standard case-control methods were used for the estimation of crude relative risks (RR) with 95% confidence interval. Multivariate unconditional logistic regression was used to adjust for potential confounding factors. With 66 exposed cases (17.4%) and 28 exposed controls (1.9%), allopurinol was the drug most frequently associated with SJS and TEN, multivariate RR=16 (9.1-28). Allopurinol exposure had increased since a prior study (1989-1993) for both cases and controls (5.3% and 1.0%, respectively). The mean daily doses of allopurinol were higher in cases as compared to controls (258±66 mg/day vs. 189±95 mg/day; p=0.001). The use of 200 mg or more of allopurinol daily was associated with an increased risk for SJS or TEN: 36 (17-76) as compared to lower daily doses: 3.0 (1.1-8.4). The risk period was restricted to short-term use of allopurinol (<8 weeks): RR=264 (36-) while no significant risk was found for long-term use (∝8 weeks): 1.5 (0.6-3.9). Indeed long-term users of allopurinol were more often exposed to other high-risk drugs for SJS/ TEN than short-term users (p=0.0006). Exposure to diuretics was not associated with an increased risk for SJS/TEN. Recent increase of allopurinol exposure rate in SJS/TEN cases and controls probably implies overuse and inappropriate indication.

0418

Psoriasis patients have an increased risk of lymphoma—results from a population based cohort study in the United Kingdom

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Psoriasis affects about 1-2% of the population. Psoriasis is likely a disease of antigen presentation and lymphocytes raising the possibility that its pathogenesis may also be associated with lymphoproliferative malignancies. Furthermore, therapies for psoriasis target the immune system and may have a theoretical risk of inducing lymphomas. We performed a cohort study to assess the risk of lymphoma in psoriasis patients using the General Practice Research Database (GPRD). GPRD is a medical records database and contains more than 35 million person years of follow-up. The GPRD has been shown to validly capture data on a variety of medical conditions including lymphoma. The population studied was a 10% sample of patients 65 or older examined between 1988-1996. First, we validated our ability to ascertain psoriatics by showing that 95% of GPRD patients with psoriasis receive treatments consistent with this diagnosis and that the frequency of psoriasis in the GPRD population is consistent with other population based studies. Subsequently we developed cohorts of 2,718 patients with psoriasis and 105,203 patients without psoriasis (reference population). Median follow up time was 46 months. We observed 276 lymphomas. Psoriasis patients had a 2.95 relative rate of developing lymphoma (95% CI 1.82, 4.76) as compared to those without psoriasis. This estimate did not change if we controlled for age and sex using Cox multivariable proportional hazards modeling. The rate of lymphoma changed little if we excluded mycosis fungoides (N=30) from our lymphoma diagnosis. This study suggests that the GPRD can be an important population based database to study psoriasis patients. Our results add to the growing literature demonstrating an association between psoriasis and lymphoma. Additional studies are necessary to determine if the increased rate of lymphoma is related to psoriasis severity, psoriasis treatment, or an interaction between these risk factors.

0419

A sun awareness pilot project

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Skin cancer is the most common cancer in the United States today, it is also the most preventable. Sun overexposure results in approximately 90% of all skin cancers. Americans have a one in six lifetime risk of developing skin cancer. The purpose of this pilot project was to increase the level of sun protection knowledge and awareness in parents of six-month old infants. This pretest/posttest study was modeled after the Australian Cancer Council's "Slip! Slop! Slap!" campaign. Self-reported baseline data about sun protective measures used by parents for themselves and their children was obtained from five pediatric clinics. In addition, knowledge, attitudes, and beliefs about sun and sun protection were investigated. The results showed an overall increase in parental sun protective knowledge and awareness. Most of the respondents were female, married, age 31 to 40, college educated, had one child, and had annual household incomes of $21,000. The ethnic distribution included 86% Whites, 9% Hispanics, and 5% Blacks. Skin prototype used to estimate skin cancer risk, placed 9% of the participants in the high risk group. Parents protected themselves more on average, than they did their children. Pre-intervention 81.8% of parents used sunglasses, but they did not practice or encourage this behavior to the same degree in their children. The most frequently used sun protective meaures for both parents and their children were sunscreen, sunblock, avoidance of the midday sun, and seeking shade. In conclusion, post-intervention a significant number of participants made behaviroal changes and were more aware of the risks of UV damage to the skin. Demographic characteristics cannot be generalized to the population at large because they were skewed to highly educated Caucasians with high incomes. Although parents still generally inadequately protected their children form sun overexposure, protection of children is an important responsibility for parents, guardians, and physicians and should ideally become an everyday habit for everyone.

0420

The National Registry for Ichthyosis and Related Disorders—health related quality of life and patient reported outcomes

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Health related quality of life and patient reported outcomes were analyzed from questions (modified by permission from two published, validated instruments and additional, non-validated questions) embedded in the National Registry for Ichthyosis and Related Disorders. Indices provide scores from 0 to 30, 30 being the most severe. Previous studies of adult dermatology outpatients in the UK have determined an overall score of 7.3±6.3 (mean±SD) (unaffected, 0.5±1.1). Children registered an overall score of 5.1±4.9 (normal children, 0.4±0.7; children with other chronic diseases, 0.7±2.5). Information is available from 489 Registry enrollees. Data can be viewed for all individuals, or in terms of disease diagnosis or severity of disease. The overall scores suggest Registry patients were affected to a similar degree as the UK reference populations. Subjects ∝14 years old scored 7.6±6.2; subjects •14 years old, 5.0±3.8. Scores were highest for those with the diagnosis of BCIE/EKH, CIE/LI, Darier Disease, ichthyoses with systemic findings, and Netherton Syndrome. The score for adults treated with systemic retinoids (used as a surrogate measure of disease severity) was 9.0±6.3, while those not treated with systemic retinoids scored 6.5±5.9; children treated with systemic retinoids scored 7.6±4.7, while those not treated with systemic retinoids scored 4.8±3.7. Little effect (scale 0-3) was seen on choice of career (0.9±1.1) or the ability to find childcare for affected children (0.3±0.7). Many were dissatisfied with their initial diagnosis and treatment plan (1.6±0.9), but satisfaction improved with subsequent consultations (1.1±0.6). Most subjects considered themselves in excellent, very good, or good general health. The similarity in score between UK dermatology outpatients and this population was unexpected and may reflect the more acute nature of the UK population. These data illustrate the value and complexities of patient reported information.

0421

Decreased incidence of malignant melanoma in Hungary between 1997-2001

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In order to assess the possible impact of ozone depletion on detection rates for melanoma inncidence, a frame of reference for the epidemiology of melanoma was established. Incidence rates were defined as the number of newly diagnosed primary cutaneous melanoma per 100.000 inhabitants, adjusted to the European population (European Standard Rate, ESR). Retrospective investigation of all histologically verified melanomas diagnosed in Hungary in the period 1997-2001 was performed. A total of 2332 cases were analysed. The age-adjusted incidence decreased in the period from 4,7 in 100.000 to 3,3 in 100.000 (from 4,3 in 100.000 to 3,3 in 100.000 for men and from 5,1 in 100.000 to 3,5 in 100.000 for women). Mortality rates remained largely unchanged, around 2 in 100.000. These data, together with our previously presented regional melanoma data (1989-1998) suggest that the short term trend for melanoma incidence in Hungary in the observed period is probably influenced by the 8-11 years cyclical activity of the Sun. These findings also mean, that the variability of UVB radiation within a cycle overcomes the possible UVB increase due to ozone depletion. Long term analysis of baseline incidence is not possible, because it needs retrospective melanoma data of at least three Sun cycles.

0422

Tanning device usage: what are parents thinking?

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Given the increasing prevalence of tanning device usage among adolescents, our aim was to determine parental knowledge about indoor tanning and whether this affects their practices regarding tanning device usage by their children. A survey was administered to 283 consecutive parents of 516 children attending an outpatient clinic. The survey consisted of three sections: family demographics; parental knowledge of the risks, regulations, and prevalence of tanning device usage by minors; and past and current tanning device usage by the parent and their children. Parents were also asked at what age they would allow their children to use tanning devices in the future. Results were summarized with descriptive statistics and Fisher's exact tests using STATA. Of the 255 parents who completed the majority of the quiz, 162 parents scored ≤70%. Only 16.7% of parents were aware of the limited amount of sunburn protection provided by tanning devices, and 38.3% did not know that tanning device exposure can be more harmful to adolescents than adults. Other frequent misconceptions were that federal and state regulations limit tanning device exposure by minors (37.9% and 31.8%, respectively). Lower scores on the knowledge quiz did not predict current or past tanning device use by their children since tanning device usage incidence was 2.8% for children of parents scoring <70% and 6.3% for parents scoring 70% (p=.172), though the predictive power of the knowledge quiz was limited by the small number of children (25/516) reported to have ever used tanning devices. Most parents (71%) never wanted their child to use tanning devices, regardless of age, but only 13.4% of parents had ever talked to their child about tanning devices. These results suggest the need for more education to increase parental knowledge and awareness of the indoor tanning trend among adolescents, which may ultimately help them to initiate discussion with their children.

0423

Validity of patient self-reported history of skin cancer

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Information regarding a patient's past medical history is typically obtained through patient self-report. However, to the best of our knowledge, no study to date has investigated the validity of using this method for obtaining a medical history of skin cancer. We compared the responses to a mailed survey asking about skin cancer history with the results of chart review for 300 patients seen in the Department of Dermatology at the University of Pennsylvania between January 1998 and June 2001. Two hundred of the 300 patients were randomly selected from those with ICD-9 documentation of non-melanoma skin cancer, while the other 100 were randomly selected from those with ICD-9 documentation of warts, rosacea, dermatophytosis, or seborrheic keratosis. Patients were considered to have chart documentation of skin cancer if the medical record contained either a confirmatory pathology report or a report from Mohs micrographic surgery documenting a malignancy within the Mohs specimen. After an exhaustive search, charts were located for 258 (86%) of the 300 eligible subjects. 188 (72.9%) of the 258 subjects with locatable charts had chart documentation of skin cancer. Using chart documentation as the gold standard, patients correctly identified their basal cell carcinoma status in 84.3% of cases; their squamous cell carcinoma status in 81.5% of cases; their overall non-melanoma skin cancer status in 91.8% of cases; their melanoma status in 94.8% of cases; and their overall skin cancer status in 92.6% of cases. Men were more likely than women to give their correct overall skin cancer status (OR=3.60; 95% CI=1.32, 9.77). Those younger than 65 years of age were more likely to give their correct squamous cell carcinoma status (OR=2.45; 95% CI=1.25, 4.79) or melanoma status (OR=3.61; 95% CI=1.04, 12.41), although there was no significant difference in the likelihood of their giving the correct overall skin cancer status. Patient self-report appears to be a valid method for obtaining skin cancer history information.

0424

Comparisons of efficacy and cost-effectiveness of topical immunomodulators in the management of atopic dermatitis

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Background: The availability of new topical immunomodulators greatly improves treatment options for patients with atopic dermatitis (AD). Objective: To propose ways to compare the efficacy and cost-effectiveness of tacrolimus ointment and pimecrolimus cream as monotherapy for the treatment of AD patients with moderate disease who are not responsive to or not well-controlled with topical corticosteroids. Methods: Comparisons were made between Eczema Area and Severity Index (EASI) scores, and converted to disease controlled days (DCDs) on treatment. Results: A comparison of EASI scores for tacrolimus and pimecrolimus found that pimecrolimus was approximately 60 % as efficacious as tacrolimus after two to four weeks of treatment. Based on these efficacy rates, average cost-effectiveness ratios were estimated at $7.34 per DCD for tacrolimus and $11.34 per DCD for pimecrolimus. Sensitivity analyses found that pimecrolimus would be cost-effective only if its efficacy approached 90 % of tacrolimus efficacy, or if the costs of the products were significantly different than current AWPs. Conclusion: We estimate that the efficacy of pimecrolimus to be 60 % of the efficacy of tacrolimus and therefore, tacrolimus to be more cost-effective for the treatment of moderate AD. Sensitivity analyses revealed that pimecrolimus cream would need to be 90 % as efficacious as tacrolimus ointment in order to be cost-effective in this patient population.

0425

Dysplastic nevi in persons with malignant melanoma: a matched case-control study

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The relationship between dysplastic nevi and malignant melanoma is debated. We sought to learn if atypical nevi in persons with a new diagnosis of malignant melanoma demonstrated histologic dysplasia to a greater extent than atypical nevi in spousal controls. Patients were accrued into the study following a new diagnosis of malignant melanoma. Full cutaneous examinations were performed, and the single most clinically atypical macular nevus biopsied in both cases (n=81) and controls (n=81). In addition, demographic and sun-related behavior data were obtained. The biopsies were evaluated by a single dermatopathologist and graded on histologic dysplasia (0=no dysplastic features, 1=mild dysplasia, 2=moderate dysplasia, 3=severe dysplasia). Odds ratios were calculated by conditional logistic regression after adjustment for age, Fitzpatrick skin type, and number of blistering sun burns. Dysplasia was a relatively common histologic finding, occurring in 119 (70%) of study persons. All dysplastic nevi were graded as either mild (n=113) or moderate dysplasia (n=6). Mild dysplasia was not associated with melanoma (adjusted OR 1.01, 95% CI 0.48-2.14) when compared to nevi with no dysplastic features. Moderate dysplasia did appear to be related to melanoma, although this was not a statistically significant association (adjusted OR 2.29, 95% CI 0.39-13.59). Our findings suggest that mild dysplasia is a common histologic finding and does not occur more commonly in nevi of persons with malignant melanoma.

0426

Effect of personalization and candy incentive on response rate for a mailed survey of dermatologists

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Many techniques have been proposed to increase the response rate of a survey study. We investigated the effect of incentives and personalization on the response rate to a mailed survey on issues surrounding melanocytic lesions that was sent to 800 members of the American Academy of Dermatology. A modified version of the Dillman Total Design Method was used as the protocol for mailing. Potential subjects were randomly assigned to one of four types of initial mailings: one with a "personalized" hand-written note on the cover letter and a hard-candy incentive; one with a "personalized" note only; one with a candy incentive only; and one with no additional materials. Additionally, half of the individuals in each group were randomly chosen to receive a follow-up reminder postcard with a hand-written signature, while the other half received a reminder postcard containing a pre-printed signature. Eighteen (2.25%) of the potential 800 subjects did not receive the survey because they had died or had been assigned an incorrect address, and those individuals were excluded from analysis. Responders were defined as those who either completed the survey or informed us that they would not be participating. 61.3% (241/393) of those receiving a "personalized" note responded compared to 51.6% (201/389) of those not receiving notes (p=.006). 57.4% (224/390) of those receiving candy responded compared to 55.6% (218/392) of those not receiving candy (p=.607). 60.0% (234/390) of those who received a reminder postcard containing a hand-written signature responded compared with 53.1% (208/392) of those receiving a postcard with a pre-printed signature (p=.050). Our results suggest that including a "personalized" note in the initial mailing and using hand-written signatures on the reminder postcard may be effective methods for increasing the response rate to a mailed survey, but that candy incentive is not effective.

0427

Surveillance variation in Medicare beneficiaries after excision of nonmetastatic melanoma

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We evaluated national surveillance variation after local excision of melanoma. Using Surveillance, Epidemiology, and End Result (SEER)-Medicare linked data, we identified white Medicare eligible patients >65 years diagnosed with local or regional stage incident invasive melanomas during 1992-1996 and no previous cancer. The 3,317 patients had a median follow-up of 3.5 years. Surveillance data were obtained from Medicare claims from 6 months following diagnosis through 1998 or censorship at subsequent cancer diagnosis or death. The percent of patients receiving at least one surveillance test was: 94% for skin examination, 92% for chest x-ray, 24% for Chest CT, 33% for abdominal/pelvic CT, 32% for abdominal ultrasound, 33% for head CT, 92% for liver enzyme, and 94% for CBC. Of the tested patients, the median number of tests performed was: 8 for skin examination, 8 for chest x-ray, 3 for chest CT, 4 for abdominal/pelvic CT, 2 for abdominal ultrasound, 3 for chest CT, 7 for liver enzymes, and 8 for CBC. Chi square analysis revealed significant ($p<.05$) variation by SEER region for all tests except for chest CT. Men were more likely to receive at least one chest CT, abdominal/pelvic CT, and abdominal ultrasound, while women were more likely to receive skin examinations. Older patients were more likely to receive surveillance testing. These figures correspond to up to 2-fold or 25% absolute differences in the percent of patients receiving surveillance. As expected, the proportion of patients tested increased with increasing tumor Breslow depth. Analysis of Variance revealed that patients receiving at least one surveillance test generally did not vary as significantly by SEER region, gender, or age in the mean number of tests received. We conclude that there is substantial national variation in the presence of melanoma surveillance by geographic area and patient characteristics, and that patients who do receive surveillance testing undergo moderately large differences in follow-up testing regimens.

0428

Risk of infections in relation to drugs intake for the induction of toxic epidermal necrolysis and Stevens Johnson syndrome

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Anti-infective drugs have a high suspicion to induce TEN and SJS. HIV-infected individuals also have an increased risk. The question is whether other infections than HIV are associated with an increased risk of TEN and SJS or that the antibiotics given for these infections are the causative agents. Alternatively, infections could influence the risk of TEN and SJS by modulating antigenicity of anti-infective or other suspected drugs. The EUROSCAR group (European group to study Severe Cutaneous Adverse Reactions) used 379 validated cases of TEN or SJS and 1505 controls to address this question. Drugs and infection history were collected. Relative risks were estimated and multivariate logistic regression was used to adjust for potential confounders. Stratified analyses among different drug groups were performed to calculate the impact of infections on the risk of TEN and SJS. Although infections were associated with an increased risk of TEN and SJS with a multivariate relative risk of 2.0 (1.5;2.8), this risk was low compared to the risks caused by HIV infection: 10 (2.3;43) or by antibiotics: 8.0 (2.1;20). Stratified analyses showed no increased risk for infection: 0.8 (0.3;2.2) in the individuals who were using anti-infective drugs. Infections were associated with a slightly but not significantly increased risk in individuals who were using highly suspected drugs (other than anti-infectives) with a relative risk of 2.1 (0.8;5.3) or drugs with a low or moderate risk: 1.7 (1.0;2.8). Infections other than HIV do not appear to impose a significant risk for TEN and SJS in contrast to e.g. erythema multiforme with or without mucosal involvement. The anti-infective drugs given for these infections appear to be the causative agents. It cannot be excluded, however, that infections could influence to a moderate extent the risk of induction of TEN and SJS imposed by other drugs than anti-infectives.

0429

Stevens-Johnson syndrome and toxic epidermal necrolysis: the risk of antiepileptic drugs

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SJS and TEN are rare but life-threatening severe cutaneous adverse reactions (SCAR) to various medications, among them antiepileptic drugs (AED). The following analysis is based on data ascertained in the Euro-SCAR-study (case control surveillance of SCAR in Europe) comprising information on 379 cases and 1505 controls between April 1997 and Dec. 2001. Compared to the results of the former SCAR-study (1989 - 1993) an increased multivariate relative risk (RR) of 61 (CI 10-190) was found for carbamazepine, a risk of 14 (3.3-56) for phenytoin and a risk of 18 (5.9-57) for phenobarbital. In contrast, the multivariate RR for valproic acid was 2 (0.6-7.4) in the EuroSCAR-study compared to 8.3 (1.8-40) in the SCAR-study. The univariate RR for lamotrigine was infinite (14-inf.), as 14 cases but no controls were exposed. Comparing the exposure of certain AED in different countries, the RR for carbamazepine was substantially higher in France and Germany compared to Austria, Israel, Italy, and The Netherlands. Lamotrigine showed a lower rate of exposure among cases, valproic acid among cases and controls in Germany compared to other countries. The time-latency between beginning of AED-use and onset of SCAR revealed a median of 15 days for carbamazepine, 19.5 days for lamotrigine, but more than 365 days for valproic acid. The comedication with highly suspected drugs was 75% for valproic acid but about 15% for other AED. In addition, the amount of comedication with another AED was esp. high for lamotrigine and valproic acid (9/14, and 9/16 resp.). These 9 patients did not differ in terms of severity and time-latency, whether they followed a lead-in period for lamotrigine or not. The risk profile of lamotrigine seems to be comparable to that of carbamazepine and there is no increased risk for valproic acid to induce SCAR.

0430

Development and validation of a health-related quality of life instrument for women with melasma

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Melasma can have significant emotional and psychological effects on those affected with the condition. Our purpose was to develop and validate a disease-specific HRQoL instrument to identify the areas of the patient's life most impaired by melasma as well as the effects of the condition on their level of functioning in correlation with disease severity, the Melasma Quality of Life scale (MELASQOL). A random sample of 102 women with clinically diagnosed melasma were evaluated by the investigator using the Melasma Area and Severity Index (MASI). These patients were then anonymously surveyed using standard skin-disease HRQoL measures and a specially developed skin discoloration evaluation questionnaire. The 10-item MELASQOL scale was devised from these components. The psychometric properties of the MELASQOL were comparable with the properties of the DLQI and the SKINDEX-16. The discriminatory ability of the MELASQOL is superior to the SKINDEX-16 and the DLQI for melasma. The three life domains most adversely affected by melasma (social life, recreation/leisure, and emotional wellbeing) were highlighted by this instrument. The high correlation with the DLQI, the SKINDEX-16, and the skin discoloration questionnaire suggests that the new scale is a valid instrument, which can be used to monitor the level of impairment individuals suffer due to their melasma.

0431

Correlation of Breslow depth of melanoma and extent of tanning bed exposure as a measure of dose-response relationship?

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Recent epidemiologic studies have suggested a potential association between tanning bed (TB) exposure and cutaneous melanoma. Because most studies have focused on ever-use of TB, a possible relationship between the extent of TB exposure and risk of melanoma has yet to be elucidated. To further examine the relationship between extent of TB use and melanoma, surveys assessing extent of TB exposure were collected from academic dermatology clinic patients (n=1518). Medical records were reviewed, and the Breslow depth obtained if applicable. A Pearson correlation coefficient was computed to assess possible linear association between the extent of TB exposure (calculated as the product of self-reported number of TB exposures and the average time spent per TB session) and the Breslow depth of melanoma. Among patients surveyed, 32.1% reported a history of TB exposure. Admitted TB users with melanoma reported greater average time spent per TB sessions, with 47.8% reporting sessions greater than 20 minutes compared to 22.0% among TB users with no history of melanoma ($p<0.01$). Similarly, admitted TB users with a history of melanoma were more likely to report greater than 20 TB exposures compared to TB users without melanoma, though not significantly so (62.1% vs. 47.3%, $p=0.12$). Furthermore, there is a positive though statistically insignificant correlation between Breslow depth and overall TB exposure in minutes (n=23; Pearson $r=+0.18$, $p=0.48$). This study offers the first published attempt to examine a possible linear dose-response relationship between the extent of TB exposure and risk of the development of cutaneous melanoma. Although the positive correlation between the depth of cutaneous melanoma invasion and the estimated lifetime TB exposure is not statistically significant, a larger epidemiologic study is needed to clarify this relationship given the solemn public health implications of this popular activity.

0432

Diagnosis, treatment, and management of melanoma *in situ* by US dermatologists

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To assess current beliefs and practices of US dermatologists regarding the diagnosis, treatment, and management of melanoma in situ (MIS). Methods: A survey was mailed to a random sample of 1200 practicing US dermatologists. Results: Response rate was 54%. 88% of responders believe that MIS is a malignancy and 11% believe it is a premalignant condition. 30-48% stated that they did not know whether untreated MIS would progress to invasive metastatic melanoma and 23-36% stated that all MIS would progress. In the clinical evaluation of suspect lesions, 31% used no diagnostic tools, 59% used magnifying lens, and 18% used dermatoscope. 55% of dermatologists diagnose <10 MIS cases/yr while 8% diagnose >30 cases/yr. The preferred biopsy method was excisional (39%), saucerization (34%), and incisional (punch/shave) 27%. 57% of responders sent all pigmented lesions directly to a dermatopathologist. Responders estimated that approximately 60% of cases clinically suspect for MIS were confirmed to be MIS on pathology. For clinically suspect lesions diagnosed on pathology as "benign", 30% of dermatologists accepted the diagnosis and 70% pursued additional pathology review. Regarding therapy, approximately 50% performed definitive excision in >75% of cases in their office. 18% of dermatologists referred all cases to other physicians for definitive excision. Regarding laboratory evaluation and follow-up, 77% of dermatologists do not order lab tests/X-rays. After the first year of MIS diagnosis, 92% of dermatologists recommend skin screening and follow-up exams every 3-6 months. Conclusion: Most respondents identified MIS as a malignancy, however, apparently much confusion still surrounds the diagnosis and management of MIS. In addition, most MIS cases are excised in dermatologists' offices and this may have implications in accurately calculating MIS incidence rates.

0433

Risk of melanoma is associated with tanning bed exposure, particularly among young females

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Although deleterious effects of tanning bed (TB) exposures have been demonstrated at the molecular level, the epidemiologic association between the development of melanoma and TB exposure is less than absolutely definitive. Previous literature has sporadically considered only a few possible confounding factors, including sun sensitivity, sun exposure, and socioeconomic status. To further explore the relationship between melanoma and TB use, surveys assessing TB exposure and sun protection practices were collected from academic dermatology clinic patients (n=1518), and review of medical records was performed. Nearly one-third of patients had visited a TB (n=487). Women 45 years of age and younger accounted for about 60% of all TB users, with 59.4% of these women reporting a history of TB use compared to 24.5% of women over the age of 45. After controlling for confounding factors, including indoor vs. outdoor occupation and leisure activities, Fitzpatrick skin type, history of blistering sunburn, use of sunscreen and sun protective clothing, history of phototherapy, and level of education, ever-use of TB became a significant risk factor for the development of melanoma ($p<0.05$;OR=1.83;95% CI=1.07-3.14). Among women 45 years of age and younger, ever-use of TB was an even greater risk factor for the development of melanoma after controlling for the same confounding factors ($p<0.05$;OR=3.25;95% CI=1.03-10.28). Furthermore, female tanning bed users 45 and younger with melanoma tended to report more TB sessions ($p<0.01$) and more time per session ($p<0.01$) than those with no history of melanoma. These results suggest that a history of TB exposure is a significant risk factor for melanoma, especially among women 45 and younger, even after controlling for confounding factors. These findings reinforce the public health urgency to increase awareness of the potential health hazard of TB exposure, particularly among young females. Further studies are needed to clarify whether a dose-response relationship exists between the extent of TB use and the risk of melanoma.

0434

Is a high potency corticosteroid needed with the antifungal agent? An analysis of factors influencing clotrimazole/betamethasone diproprionate prescriptions among US outpatient physicians

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Despite concern associated with the necessity of an additional fluorinated, high potency topical corticosteroid, the clotrimazole/betamethasone diproprionate combination remains a frequently prescribed topical agent in the U.S. This research was performed to better understand the physician and patient characteristics associated with the prescription of this combination medication in outpatient settings. Data from the National Ambulatory Medical Care Survey (1990-2000) were used to determine the patient and physician factors associated with a prescription for clotrimazole/betamethasone diproprionate. The most common diagnoses of patients treated with the drug were also determined. Family medicine physicians were more than twice as likely (OR: 2.61, 95% CI: 1.59, 4.30) and internists were more than 3 times as likely (OR: 3.52, 95% CI: 2.07, 5.97) to prescribe clotrimazole/betamethasone diproprionate compared to all other physicians when faced with a dermatologic diagnosis. Prescription rates of the combination medication were higher among patients of non-white race (OR: 1.55, CI: 1.07, 2.25). The increased risk of potentially inappropriate prescription of clotrimazole/ betamethasone diproprionate by primary care physicians and increased probability of medication receipt in racial minorities is of concern, when safer alternatives such as antifungals or anti-inflammatory medications without the side effects of the combination medication are easily available.

0435

Evidence for a founder mutation in the fumarate hydratase gene in patients with multiple cutaneous and uterine leiomyomata from Iran

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Multiple cutaneous and uterine leiomyomata (MCL) syndrome is an autosomal dominant disease characterized by the presence of concurrent benign tumors of smooth muscle origin (leiomyoma) in skin and uterus. MCL can also be associated with renal cell cancer (HLRCC). The genetic locus for MCL and HLRCC was recently mapped to chromosome 1q42.3-43 and subsequently, dominantly inherited mutations in the fumarate hydratase gene (FH) were identified. Importantly, the analysis of the FH gene in tumors of MCL patients revealed a second mutation inactivating the wild-type allele in some of the tumors. Based on these findings, it has been suggested that FH may function as a tumor suppressor gene in MCL. Previously, we reported different types of mutations in MCL patients including missense, nonsense and frameshift mutations. Here, we report the identification of the splicing mutation 905-1G>A in four families of Iranian origin. The mutation occurs at the invariant G of the 3' acceptor splice site (AG) of intron 6. The analysis of highly polymorphic markers in the vicinity of the FH gene showed a shared haplotype in these four families, suggesting that 905-1G>A represents a founder mutation. These results will allow for direct screening of the 905-1G>A mutation in affected MCL families and sporadic patients of Iranian descent.

0436

Patients with palmoplantar psoriasis have more physical disability and discomfort than patients with other forms of psoriasis: implications for clinical practice

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Psoriasis is a chronic, unpredictable and incurable disease that has a negative impact on patients' quality of life. Palm and sole psoriasis can add to this negative impact as it directly affects activities of daily living. This study estimated the prevalence of palmoplantar psoriasis in a patient population and to describe the added impact on patient outcomes. 317 individuals with psoriasis completed a comprehensive assessment battery. Patients with palmoplantar psoriasis (n=124, 39%) were compared to patients without palmoplantar involvement with respect to functional disability, psychiatric symptoms, physical and social discomfort, self-reported psoriasis severity and HRQL. The study found that patients with palmoplantar involvement reported significantly greater physical disability and physical discomfort than patients without palmoplantar involvement (both $p<0.01$). There were no differences between the two groups with respect to psychosocial outcomes. Patients with palmoplantar psoriasis are affected to a greater degree by the physical aspects of the disease than patients without palmoplantar involvement.

0437

Atrichia with papular lesions in a series of non-consanguineous families defined by mutations in the hairless gene

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Atrichia with papular lesions (APL) is a rare, recessively inherited disorder that results in complete loss of scalp and body hair due to underlying mutations in the hairless gene. APL is easily confused with the much more common complex genetic trait alopecia universalis, which has an unknown genetic basis. Despite this clear demarcation at the molecular level, the two conditions can be indistinguishable clinically. However, diagnostic criteria for APL have recently been delineated that allow easy clinical distinction from alopecia universalis. These cardinal features of APL include: hair that was shed early and never regrew, associated papular eruptions and complete lack of response to all attempted treatments for alopecia universalis. Three families of Iraqi-Moroccan, Australian (of English descent) and Russian descent with complete alopecia were recently ascertained, all of whom had a single affected proband within a small nuclear family that denied any history of consanguinity. In each of the probands, we identified compound heterozygous mutations on distinct alleles of the hairless gene, thereby confirming the diagnosis of APL by molecular analysis. These findings stress the importance of inclusion of APL in the differential diagnosis of alopecia universalis, particularly in families considered to be of low risk due to the lack of parental consanguinity. Accurate diagnosis of APL is easily established by application of the diagnostic criteria and by mutation analysis in the hairless gene. Moreover, discrimination between the two conditions allows for the sparing of an aggressive and unnecessary alopecia universalis treatment regimen for APL patients.

0438

The effect of gender on workforce patterns of recent dermatology program graduates

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There currently appears to be a shortage of dermatologic services in the U.S., especially in medical dermatology. Some have questioned whether the number of recent trainees practicing part-time is contributing to this worsening shortage, and whether gender affects this choice. To address this question, an anonymous survey was distributed to recent dermatology program graduates taking a board exam review course in 2000. Women reported seeing patients an average of 25 hours per week (hrs/wk) compared to 31 hrs/wk reported by men ($p= 0.003$). 43% of men had children compared to 34% of women. The differences between genders was also affected by having children ($p=.005$). Women who had children tended to see patients less often than women without children (23 hrs vs. 27 hrs), but men with children saw patients about the same as men without children (31 hours vs. 30 hours). The breakdown of hours spent in differing areas of dermatology practice showed men spent an average of 14 hrs/wk doing Mohs procedures while women only spent 2 hrs/wk ($p< 0.001$). Men spent two hours more per week doing non-Mohs dermatologic surgery compared to women (7 hours vs. 5 hours, $p= 0.003$). Finally, men also spent more time performing cosmetics procedures than women (4 hrs vs. 2 hrs, $p= 0.020$). Survey results suggest that women newly entering the workforce are working fewer hours then men. However, the actual hours spent practicing medical dermatology, where the shortage of services is most acute, is roughly equal between genders because men spend more hours practicing surgery and cosmetic procedures. According to this survey, both men and women ranked location as the most important consideration when selecting a job. Questions that still need to be addressed include how many actual patients per hour are seen, and whether these work patterns seen early in a dermatologist's career is representative of trends in the general workforce.

0439

The role of fellowships in workforce patterns of recent dermatology graduates

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In light of the current shortage of medical dermatologic services in the U.S., concern has developed about the number of trainees choosing surgical fellowships and other non-medically oriented-practices. To explore the choices of dermatologists entering the workforce, an anonymous survey was distributed to dermatology training program graduates taking a board exam review course in 2000. 204 people responded to the survey, capturing 53% of the 2000 residency graduates (157) and a substantial number of fellows (42). At the time of the survey, 33 respondents (18%) were in a fellowship program while 9 respondents (5%) had just completed one. 18 fellows (42%) participated in a Mohs fellowship, the most popular. Bench research fellowships attracted 7 fellows (16%) and were second in popularity. 4 (9%) respondents each were in cosmetic, pediatric dermatology, or dermatopathology fellowships. While women comprised the majority of respondents (55.7%), male respondents were more likely to have either completed or be currently employed in a fellowship (32%) than women (17%, p=0.06). 45% of those completing or starting fellowships had student loan debt while 60% of those not involved in fellowships had student loan debt. The average debt for those completing fellowships, starting fellowships or not involved in fellowships was $57,000; $79,000; and $77,000 respectively. Half of those finishing a fellowship went into an academic practice. Differentiating factors between graduates who chose to work in an academic practice (27) and those who did not (136) included their increased emphasis on the importance of research ($p<0.001$) and teaching ($p<0.001$) opportunities. Interestingly, debt level did not differ between those going into academic practice and those who did not. In conclusion, a substantial number of trainees choose to pursue fellowships, prolonging a significant debt burden and delaying entry into the workforce. Second, the large number of people training in Mohs makes it unlikely that they will be able to practice Mohs full-time. Lastly, bench research is still attracting a significant number of fellows.

0440

The accuracy of computer-assisted digital epiluminescent microscopy versus dermatologists in the evaluation of pigmented lesions

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We evaluated the accuracy of recently developed computer-assisted digital epiluminescent microscopy (CADEM) in evaluating pigmented lesions and assessing the need for a biopsy. At a university dermatology clinic, pigmented lesions were scanned by CADEM and evaluated by a dermatology attending, then biopsied. Using the histopathologist's evaluation as the gold standard, the accuracy of the CADEM was compared to that of the dermatologist. We evaluated 127 lesions in 77 patients, of which 107 lesions were included in the study. We excluded non-nevomelanocytic lesions. Lesions were evaluated by both CADEM and dermatologists blinded to CADEM results, using a color-coded evaluation scheme. Green indicated a biopsy was not necessary (e.g. normal nevus), yellow indicated that a biopsy should be considered (e.g. nevus with architectural disorder and/or atypia), and red that a biopsy was definitely indicated (e.g. melanoma). All evaluations rated as yellow or red by the histopathologists were considered positive and all evaluations rated as green were considered negative. Dermatologists had a sensitivity (SEN) of 81%, specificity (SPE) of 35%, positive predictive value (PPV) of 46%, and negative predictive value (NPV) of 74%. CADEM had a SEN of 65%, SPE of 54%, PPV of 49%, and NPV of 74%. Using any positive evaluation by either CADEM or dermatologists but ignoring negative CADEM evaluations resulted in increased SEN (88%), decreased SPE (28%), and a similar PPV (46%), and NPV (78%). Dermatologists had a higher sensitivity, lower specificity, and similar predictive powers compared to CADEM. Using a positive CADEM result to convince one to perform a biopsy, but not allowing a negative CADEM to dissuade one from doing a biopsy could enhance dermatologists' sensitivity. Such technology, especially if better accuracy were achieved, could have significant implications for melanoma screening.

0441

Comparison of skin biopsy triage decisions by store-and-forward teledermatology and face-to-face dermatology in 49 patients with pigmented lesions and neoplasms

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The present study aimed to determine the relative efficacy of store-and-forward teledermatology versus face-to-face dermatology in triaging whether growths that a primary care physician finds concerning for neoplasia warrant a biopsy. A primary care physician prospectively enrolled patients between July 10, 1998 and August 4, 2000. This physician acquired images with an Olympus D-600L digital camera. These 1.4 mega pixel images were stored in a compressed (4:1) Joint Photographic Experts Group format, downloaded to a personal computer, and transmitted to a teledermatologist by electronic mail through a private encrypted network. Clinical history and reason for consultation accompanied the images. The patients were simultaneously scheduled to see a face-to-face dermatologist. After assessing the case, the teledermatologist notified the primary care physician of whether a biopsy was indicated. The face-to-face dermatologist would open an envelope containing the teledermatologist's biopsy decision after he had made his own, in this way ensuring that he was blinded to the teledermatologist's biopsy vote. A biopsy was performed at that visit by the face-to-face dermatologist if either he or the teledermatologist voted for biopsy. Sixty-one subjects (33 male) were enrolled. Of the 49 evaluable patients, the face-to-face dermatologist and teledermatologist recommended a biopsy for the same 26 patients, yielding a sensitivity of the teledermatologist of 1.00 (95% confidence interval: 0.87, 1.00) and a specificity of 1.00 (95% CI: 0.85, 1.00). The agreement between the two dermatologists (kappa) was 1.00 (95% CI: 0.72, 1.00). Store-and-forward teledermatology may provide an accurate and cost-effective method of determining whether to biopsy skin growths in patients seen by primary care physicians.

0442

Differences in the perceptive importance and personal practice of sun protection among primary care physicians are reflected in their clinical practice

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There is inadequate data regarding sun protection awareness, personal practice, and patient education among primary care clinicians. Anonymous surveys were mailed to staff and housestaff physicians in the departments of medicine, family practice, and pediatrics. Among 170 primary care physicians (29% response rate), 68% reported wearing sunscreen whenever they are outside, 8% did so daily, and 7% never wore sunscreen. Additionally, 32% reported wearing protective clothing consistently and 59% did so occasionally. Overall, when given a list of preventive medicine issues to rank according to importance (1=least important, 10=most important), sun protection was given the lowest relative ranking. Overall, the most important preventive medicine issue ranked by these clinicians was smoking (9.0), followed by active lifestyle (8.2), then cholesterol (7.4), and lastly sun protection (6.7). Pediatricians placed highest relative ranking of 7.7 for sun protection. Furthermore, pediatricians were the most consistent in providing sun protection education as 60% reported this practice during greater than half of the visits followed by 32% of family physicians and 17% of internists surveyed. Among staff physicians, 70% reported wearing sunscreen whenever they are outside, compared to 58% of housestaff physicians. Staff physicians reported providing sun protection counseling more frequently than housestaff physicians, 32.8% vs. 8.3% respectively. Overall, the greatest impediments in providing sun protection counseling reported by this cohort of clinicians included inadequate time during office visits, not thinking about sun protection during office visits, and lack of proper training. In short, differences in the perceptive importance and personal practice of sun protection may be reflected in the self-reported clinical practice of these primary care physicians. This reinforces the urgency and the opportunity to educate primary care clinicians to join the public health effort to increase sun protection awareness.

0443

Atrichia with papular lesions resulting from a novel homozygous missense mutation in the hairless gene

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Atrichia with papular lesions (APL, OMIM 209500) is a rare autosomal recessive disorder characterized by complete and irreversible hair loss shortly after birth. Affected individuals also develop papular lesions of keratin-filled follicular cysts over extensive areas of the body. Mutations in the hairless gene, a putative single zinc-finger transcription factor protein, have been implicated in the pathogenesis of this disorder. Here, we describe a small Italian family with two of four offspring affected with APL. The proband is a 10-year-old girl with generalized body alopecia and absent eyebrows and eyelashes at birth. In the first four years of life, she gradually lost all scalp hair and developed diffuse skin-colored papular lesions on her limbs and face. A 4-year-old sister of the proband presented with similar symptoms. In the past years, the younger sister has been shedding the remaining scalp hair, sparing centroparietal and occipital areas of the scalp. Along with alopecia, the younger sister has recently begun to develop few papular lesions on the back. Both parents and two other siblings of the proband are unaffected, and the parents denied any history of consanguinity. Sequence analysis of the hairless gene revealed a novel homozygous missense mutation in exon 5. The mutation, designated E583V, resides in between LXXLL motif of TRIPS (thyroid hormone receptor interacting proteins) in exon 5 and the six-cysteine zinc-finger motif in exon 6. Amino acid sequence neighboring the LXXLL motif and zinc-finger domain encompassing the E583 is highly conserved in human, monkey, rat and mouse hairless proteins. Further, this mutation was not identified in a large number of unaffected, unrelated control individuals, arguing against it being a polymorphism. Our findings extend the body of evidence implicating hairless gene mutations in the pathogenesis of APL, and highlights the presence of the disease in small nuclear families.

0444

The spectrum of drugs that may cause acute generalized exanthematous pustulosis is different from the one for Stevens-Johnson syndrome or toxic epidermal necrolysis

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Acute generalized exanthematous pustulosis (AGEP) is a reaction mostly caused by drugs, characterized by the acute occurrence of dozens to hundreds of small, pinhead-sized non-follicular sterile pustules. In a multinational case contol study (EuroSCAR) we could detect 96 patients that fit the criteria of this disease and could be classified as definite or probable cases according to a specially developed scoring system. A thorough history of drug intake and previous diseases was used to determine the risk for different drugs for causing AGEP by comparing them with a group of 1009 hospital controls. Multivariate relative risks (RR) for drug coalitions and single drugs were calculated adjusting for age, gender, country and the intake of other drugs with an elevated risk. For the following drugs an elevated RR for AGEP is probable (95% confidence intervals (CI) given in brackets): ampicillins RR 23 (10-53.4); quinolones RR 19.88 (4.3-91); macrolide antibacterial agents RR 29 (9.6-87.7) - 10 cases attributed to pristinamycin; diltiazem RR 15.9 (4.5-55.3); antimalarial drugs RR 12 (3.2-45.1). For sulfonamide antibacterials and terbinafine (4 cases vs. 0 controls) the lack of exposed controls does not allow to calculate relative risks but the lower bound of the 95% CI of 7.1 together with the absence of suspect comedication strongly suggests an elevated risk for these drugs. Drugs with a known elevated risk for causing SJS/TEN like allopurinol or oxicam NSAIDs were not risk factors for AGEP. The spectrum of causative drugs for AGEP thus differs from that for SJS/TEN. High risk drugs for AGEP seem to be: pristinamycin, sulfonamide antibacterials, ampicillins, quinolones, terbinafine, diltiazem, and (hydroxy)chloroquine.

0445

Is there a marked rural/urban gradient for children with atopic dermatitis in South Africa?

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It is still unclear if a true urban/rural gradient exists for the occurrence of atopic dermatitis (AD)1. The objective of this study was to determine the prevalence of AD in Xhosa children living in the Eastern Cape (rural), informal settlements around Capetown (periurban) and in urban Capetown itself. This was done by conducting three cross-sectional surveys of at least 1,000 children aged 3-11 years in each of the settings using sampling frames based on local maps. Because of difficulties in translating some commonly used questions about AD into the Xhosa language, AD prevalence was determined by the presence or absence of typical signs based on the direct examination of the skin by a trained dermatologist. In all, 3,058 children participated in the study, representing an overall response rate of 99.9%. All of the children were Black African, and most were from socio-economically less advantaged communities. The point prevalence of AD according to a dermatologist was 0.7%, 1.1% and 3.7% in rural, periurban and urban settings respectively, representing an increased risk of AD if living in urban Capetown of 3.95 (95% confidence intervals 2.28 to 6.83, P<0.0001) when compared with the other locations. The risk difference could not be explained by differences in social class, age structure and age of migration between the study populations. This study provides some evidence to suggest that moving from a rural area characterised by open housing and subsistence farming to an urban location is associated with a modest increased risk of AD. Another striking finding of the study is that AD is very rare indeed in Xhosa children living in rural settlements. Further study of these communities may identify important protective factors in the environment that could lead to disease prevention.

0446

A survey of latex allergy work-place practices in US academic medical centers

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This study assesses whether U.S. academic medical centers have complied with the 1997 recommendations by the National Institute for Occupational Safety and Health (NIOSH) on latex allergy in the workplace. This is a prospective survey of all U.S. academic medical centers, containing six questions regarding latex allergy practices in these facilities. A response rate of 75% was received from the 122 academic medical centers. The results of the surveys showed the following compliance rates with NIOSH recommendations: 82% screen new employees for latex allergy; 37% screen existing employees for latex allergy; 100% have non-latex gloves available for employees; 78% have latex-allergy educational programs for employees; 62% have eliminated non-sterile powdered latex gloves from the work-place; and 25% have eliminated sterile powdered latex gloves from the work-place. This study shows that four years after the NIOSH recommendations were issued many academic centers have not complied. This is a significant issue since as many as 21% of all health care workers have developed a type I hypersensitivity to latex. Dermatologists often encounter this allergy manifesting as hand dermatitis in health care workers who wear latex gloves. However, this allergy is not limited to merely a dermatitis; those with a type I allergy are at risk for developing symptoms such as asthma, angioedema, anaphylaxis and even death from exposure to this allergen. Based upon our results, academic medical centers should be encouraged to administer screening questionnaires for employees to identify those with or at high risk for latex allergy. Additionally, further studies must be done to identify and help eliminate barriers to removing powered latex gloves from academic medical centers since the aeroallergens from these gloves are the largest source of sensitization for latex allergy in the workplace.

0447

Evaluation of a fast-track mole-check clinic at an academic dermatology office

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This study evaluated the feasibility and effectiveness of a fast-track mole-check (FTMC) clinic in an academic dermatology office. This retrospective chart review evaluated the following: length of time to appointment, type of issues addressed, and diagnsoses for patients seen during the first six months of a FTMC clinic. This new clinic was designed to provide rapid assessment of potential skin cancers. For the 172 charts reviewed from the FTMC clinic, the average length between the appointment scheduling and office visit was two weeks, compared to an 8-12 week wait in the regular dermatology clinic. Eighty-three percent of patients did not seek evaluation of secondary dermatologic issues during their clinic visit, and the most common chief complaints were changing lesions (38%) and new lesions (18%). Clincal assessment indicated 27% of patients had atypical or potentially malignant lesions, including 28 atypical nevi, 9 possible melanomas, 8 possible basal cell carcinomas and 1 possible squamous cell carcinoma. Biopsies were performed on 28% of lesions and histological findings included: 9 atypical nevi, 3 melanomas, 3 basal cell and 2 squamous cell carcinomas. Hence, 33% of biopsied lesions and 9% of all lesions evaluated were found to be skin cancer or atypical. The FTMC clinic was designed to provide patients with potential skin cancers a much faster evaluation than is available through the normal dermatology scheduling templates. Not only was this clinic able to provide a rapid evaluation of the lesions, it was successful in diagnosing and treating a significant number of skin malignancies and atypical lesions. Based upon these findings, we conclude that this FTMC clinic is both feasible and effective. Dermatology offices where patients must wait many weeks or months before getting an appointment to have a suspicious lesion evaluated should consider similar programs to increase the early detection and treatment of potentially deadly skin cancers.

0448

Impact of sulfonamide drug eruptions on 5-years survival rate of AIDS patients

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Cutaneous adverse drug reactions are frequent in AIDS patients. A 2001 publication reported a significantly shorter survival rate among patients who stopped taking cotrimoxazole for adverse events. Our aim was to determine whether sulfonamide induced eruptions had an effect on survival rate. EPITOX was a multicenter prospective study originally designed to evaluate risk factors of drug eruptions to sulfonamides in patients with AIDS. One hundred thirty-six patients hospitalized for pneumocystis carinii pneumonia or cerebral toxoplasmosis had been included from June 1995 to October 1997; 48 (36%) had experienced a drug eruption. The results of the initial study have been published : both groups were comparable for HIV viral load, prior opportunistic infections and differed only in multivariate analysis by a higher CD8 T-cells count in patients with a drug eruption. A follow-up evaluation was performed in 2002. No difference in follow-up duration was observed between the groups with and without a drug eruption (19% and 16% lost to follow-up respectively). Kaplan-Meyer overall survival rates showed only moderate differences between both groups. Actuarial two-years survival rate was 0.80 for patients with drug reaction (95% CI: 0.66-0.90) and 0.68 for patients without reaction (0.57-0.77). Five-years survival were respectively 0.72 and 0.61. By logrank test the differences were not statistically significant (chi2 = 1.32, p= 0.25). Our work is the first long-time prospective cohort study specially devoted to the evaluation of sulfonamide eruptions in AIDS patients. Even if it had not a very high statistical power, we observed no trend suggesting a deleterious effect of drug reactions in long term survival of AIDS patients.

0449

Six-month evaluation of effect of thorough skin self-examination (TSSE) intervention on TSSE practice and invasive procedures on the skin

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Monthly thorough skin self-examination has great potential for reducing melanoma mortality. The Check It Out project is a randomized trial of a multicomponent intervention designed to encourage TSSE, compared to a control dietary intervention. Participants were recruited in the practices of primary care physician volunteers. The intervention involved materials (including a videotape designed for the project), brief one-on-one counseling by a health educator at the time of the office visit, a follow-up phone call and later tailored written feedback. Assessments of outcome were by telephone interview at 0, 2, 6, and 12 months. These results are from the baseline and 6 month interviews. Of the 1359 participants randomized, 1019 (75%) completed interviews at 6 months. For this follow-up group there were slight differences (intervention vs. control) at baseline in gender (56% vs. 62% women) and mean age (53 vs. 54 years) (p=.052 and .050), but not in performance of TSSE (16% vs. 17%) or procedures performed on the skin in the prior 6 months (10.6% vs. 10.2%). However, at 6 months after intervention, 55% of the intervention group (as opposed to 35% of the control group) reported performing TSSE at least once in the prior 2 months (p<.0001). Also, a higher proportion of the intervention group reported having a surgical procedure performed on their skin after the intervention, although this was not statistically significant (8.9% vs. 6.9%, p=.2). The 6 month analyses suggest that the Check It Out intervention package is effective in increasing self-reported TSSE practice and may result in more surgery being performed on the skin. We are evaluating 12 month results and a possible modified intervention that may increase TSSE without unnecessarily increasing surgical procedures.

0450

The Minnesota Massachusetts Indoor Tanning Study: patterns of indoor tanning use among adolescents and teen access

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Indoor tanning increases skin cancer risk. In the US, there are 2-3 million teenagers customers. Determinants of use among adolescents are not known. In Minnesota (MN) and Massachusetts (MA), state regulations require parental consent for teens to tan indoors. We investigated the proportion of teens and their mothers who tan indoor and surveyed them on indoor tanning habits. We also evaluate indoor tanning business compliance with state laws on teen access. We surveyed 1298 teens, 1313 parents (90% and 75% response in each area, respectively). Ever use of indoor tanning among teens was higher in MN than MA (40.9% vs 22.1%, p < 0.0001). Among teens who tanned, 70% had started before age 16, and only half of them had asked a parent to provide written consent. Indoor tanning was significantly higher among girls, smokers, and teens who never used any sun protection. Positive attitudes toward or social aspects related to tanning were strongly associated with use. Among mothers, use of indoor tanning was highly correlated with teen use. While mothers were in agreement with their teen's report of indoor tanning use, most were not aware of laws requiring their written permission and only about half reported being very concerned if their teen tanned occasionally. In each state, a random sample of 100 tanning businesses was visited twice by 15 y.o. girls attempting to purchase a tanning session. Only 38% of Boston businesses and 55% of Minnesota businesses consistently assessed age eligibility of the teens either by asking for age or birthdate. Despite knowledge that teens were required to have parental consent, teens were able to purchase a tanning session at least once in 81% of businesses. Our data highlight the need for interventions reinforcing parents' role in limiting teen access as well as the need for strict regulations, as the majority of tanning businesses in both locations did not comply with state statutes.

0451

Differences in age, sex, and body site location of different subtypes of basal cell carcinoma: do they suggest different pathogenetic mechanisms?

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Ultraviolet radiation has been recognized as the main etiologic factor for basal cell carcinoma(BCC), whereas other exposures, such as ionizing radiation, and checmical carcinogens explain a small proportion of these cancers. Because of clinical and histological differences, different pathogenetic mechanisms have been hypothesized for subgroups of BCC. The aim ofthis study was to examine patient and tumor characteristics of 15,702 BCCs observed in 12,151 patients at the Istituto Dermopatico dell'Immacolata, Rome, Italy, between October 1991 and January 2001, by reviewing all the pathology reports. Fifty eight percent of BCCs occured in men, and 9% in patients aged 44 or younger. Interestingly, the proportion of women in the 16-44 age group were significantly higher than in the other age groups (56% vs. 40%, p=0.00). The prevailing histological type was nodular (77%), whereas 10% were superficial and 6% morpheiform. The proportion of superficial BCCs decreased with age, accounting for 19% of the tumors observed in the 0-44 age group and only for 5% of those observed among patients 75+ (p for trend = 0.00). Moreover, superficial BCC's were more common among women, and 67% of them occured on the trunk. An opposite trend was observed for nodular BCCs. The proportion of BCCs located on the head/neck region increased with age, from 56% in the younger age group to 78% in the older one (p for trend = 0.00). A reverse tendency was observed for tumors located on the trunk, which accounted for 40% of BCCs observed in patients <44 years. The findings of this study suggest that superficial BCCs represent a well defined subgroup within the clinical and histologic spectrum of basal cell carcinoma, and are likely accounted for by a different pathogenetic model, based on a different pattern of sun exposure. According to the other sources of evidences, chronic sun exposure seems to be associated to the development of nodular BCC, located in the head and neck region among older patients, whereas intermittent sun exposure, along with genetic susceptibility factors, could play a role in the occurrence of superficial BCC.

0452

Actinic keratoses: reliable assessment methods and a comparative trial between liquid nitrogen and topical 5-flurouracil

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Although liquid nitrogen (LN2) and topical 5-flurouracil (5FU) are popular therapies for actinic keratoses (AKs), no direct efficacy comparisons have been reported. Previous studies have also reported poor reliability in counting AKs. We thus conducted a prospective, quasi-randomized single-blinded trial to establish reliable methods to assess AK burden and to compare LN2 to 5FU. We recruited subjects with 2% AKs involvement of their arms from the Albuquerque, NM VA dermatology clinic. In our reliability phase, we evaluated body surface area (BSA) and direct counting (CT) of lesions > 5mm using correlations and paired mean differences between the initial and baseline (2 weeks later) visit. We analyzed data where the blinded investigators were the same (intra-rater) and different (inter-rater) at the two time points. In the efficacy phase, each subject received LN2 on one arm and 5FU on the other arm. Blinded investigators evaluated subjects at baseline and post therapy (4 months). We used an intention-to-treat analysis. 37 subjects enrolled; 2 did not finish the trial. Both BSA and CT correlated highly ($p<0.05$) and had similar mean differences between the initial and baseline visits using intra- and inter-rater data. Using intra-rater data, LN2 and 5FU demonstrated comparable efficacy in reducing AKs when measured by both assessment methods ($p<0.05$). Using inter-rater data, neither therapy effectively reduced AKs by the CT method. The BSA and CT methods have significant intra-rater reliability. BSA, more so than CT, also has significant inter-rater reliability. LN2 and 5FU are each effective in decreasing AKs; they are similar in efficacy when compared to each other.

0453

The impact of managed care on dermatology outcomes: a critical review of the literature

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Managed care, a health care delivery system utilizing a broad spectrum of cost-containing measures including limited direct access to specialists, has significantly altered health care delivery in the US over the last two decades. To assess the impact of managed care on dermatologic care, we performed a MEDLINE search and critical analysis of peer-reviewed quantitative literature published from 1980-2002. Qualifying articles mentioned 'dermatology' and 'managed care' in the title, abstract or MeSH terms, and addressed either dermatology 1) diagnostic precision and management, 2) health care utilization, or 3) outcomes analysis. Forty-three articles were identified. Eleven articles examining diagnostic precision uniformly showed significantly higher performance by dermatologists than non-dermatologists. Seven articles analyzed dermatology utilization using National Ambulatory Medical Care Survey data and reported numerous fluctuations in utilization from 1974 to 1994. Overall, patients with managed care coverage received less care from dermatologists for dermatology-related diagnoses than patients with fee-for-service payment. National Ambulatory Medical Care Survey data also showed that 39% of patients with a single dermatological diagnosis are referred to a dermatologist. Only one article (from 1983) examined the cost of care and showed similar management costs by dermatologists and non-dermatologists for dermatological disease. The results of this literature review demonstrate a paucity of quantitative peer-reviewed literature on dermatology health care delivery outcomes. National Ambulatory Medical Care Survey provides the best available data on dermatology practice, however this data has not been formally validated and appears flawed in health maintenance organization measures. Lastly, the published literature fails to address the 'backlash' against managed care currently affecting health care markets.

0454

Gender and sentinel node status affect the decision of melanoma patients to undergo adjuvant interferon Alfa-2b treatment

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Patients with thick (Breslow 4mm) primary melanomas (MM) and/or pathologic or clinical evidence of regional nodal metastasis have a high risk of tumor recurrence after surgical treatment. High-dose adjuvant interferon alpha 2b (IFNa2b) is a 1-year regimen that improves relapse-free and overall survival by approximately 10%, but has significant toxicity: 78% of patients experience severe toxic symptoms, 50% of patients require dose reduction and 23% of patients discontinue treatment before completion. The purpose of this study is to determine what patient characteristics and/or prognostic factors (Breslow level, ulceration, and sentinel lymph node (SLN) status) contribute to MM patients' decision to accept adjuvant IFNa2b. A concurrent retrospective chart and Emory Melanoma Database review identified 781 patients who underwent SLN biopsy from 1994-2001. During the study period, 135 of 781 (17.3%) patients had positive SLN or thick MM. These patients were informed of a 50% risk of recurrence and disease-related mortality, and were offered adjuvant IFNa2b. Odds ratios (OR) were estimated using multivariate logistic regression. We found that 60/135 (44%) decided to take IFNa2b while 75/135 (55%) declined. The IFNa2b group (mean age 48.8 years, 52% male, 48% female) had a mean Breslow level (mm +/-SD) 3.85 +/-2.79 with 30% ulceration and 78% positive SLN, while the non-IFNa2b group (mean age 51.9 years, 71% male, 29% female) had a mean Breslow level 4.74 +/-3.26 with 35% ulceration and 64% positive SLN. Being female and SLN positive status were strongly associated with patients who chose to take adjuvant IFNa2b (OR=2.4; 95% CI: 1.17, 5.03 p=0.017) and (OR=2.2; 95% CI: 1.01,4.97 p=0.048), respectively. Although all patients were informed that Breslow level 4mm and/or positive SLN status were associated with 50% recurrence risk, only female gender and positive SLN status were significantly associated with high-risk MM patients' decision to undergo IFNa2b adjuvant treatment.

0455

Prevalence and age-course of pigmented lesions and skin changes due to chronic UV exposure in adults

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Demographic changes and the rapid increase of skin tumor incidence indicate a need for a population-based estimate of corresponding marker lesions. Little is known, however, on the prevalence and age-course of such lesions from population-based studies. The KORA (COoperative health Research in the Augsburg region) Survey 2000 comprised a random sample stratified for age and sex of the adult population (25-74 years) of the City of Augsburg / Germany and two adjunct counties. At the study center 2323 subjects received a standardised dermatological examination (overall response 67%). We assessed the frequency (0-10, 11-50, 51-100, >100) or severity (not present, mild, moderate, severe) of the following skin signs: Common nevi (CN), Lentigines (solaris, seniles), Elastosis, actinic keratosis, dysplastic nevus. The prevalence of CN according to the categories was 31.6%, 60.3%, 6.6% and 1.5% respectively. The number of CN decreased after the 3rd decade. The proportion on subjects in the lowest category (0-10 nevi) increased correspondingly over age (25-34: 16.8%, 35-44:21.4%, 45-54:29.2%, 55-64:39.1%, 65-74:56.0%; pTrend<0.001). Lentigines solaris (36.1%, 55.4%, 67.7%, 76.4%, 80.0%), Lentigines seniles (0.2%, 3.1%, 26.5%, 63.3%, 82.5%) and elastosis (3.2%, 20.7%, 62.7%, 87.6%, 93.8%) were all significantly associated with age (pTrend<0.001). In 2.8% of the subjects an actinic keratosis was diagnosed. Similarly, a significant age-course was observed (0%, 0.2%, 0.5%, 4.3%,10.4%; pTrend<0.001). A dysplastic nevus was recorded in 5,2% of the examined persons. As for CN the number is decreasing after the 3rd decade (9.1%, 5.7%, 5.7%, 3.1%, 2.1%; pTrend<0.001). Skin signs of ageing and precursors of tumors are frequent in the adult population and follow clear age courses.

0456

Population-based assessment of cancer risk in relatives of melanoma probands

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PURPOSE: We performed a relative risk assessment for all cancers of other sites in the first-degree relatives of melanoma probands. We used the unique Utah Population Database (UPDB), which contains the genealogy of the Utah pioneers and their descendents, record-linked to the statewide Utah Cancer Registry (1966-2002). We present detailed estimates of relative risks for 30 major cancer groups in relatives of melanoma probands. METHODS: The observed cancers occurring in the 23,419 first-degree relatives of 2,901 melanoma probands were compared to expected site-specific rates of cancer using cohort-specific rates internal to the UPDB. RESULTS: 2075 total cases of all cancers were observed in the 23,419 first-degree relatives. Relative risk of all cancers in this cohort was 1.3956 (p = 0.0000, 95% confidence interval [95% CI], 1.31-1.48). A nearly 3-fold risk of melanoma was seen in first-degree relatives (p = 0.0000, 95% CI, 2.50-3.49). Cancers of the eye and pancreas, notably associated with CDKN2A mutations, were also significantly increased in this cohort. Several other cancer types with significant increases in relative risk were identified, including multiple myeloma, non-Hodgkin's lymphoma, and cancers of the kidney, stomach, colon, breast, and prostate. Similar increased risks to relatives were seen when subpopulations of high-risk melanoma probands were examined, such as age of onset before 50 years (n = 1418) and multiple primary melanoma (n = 65). CONCLUSIONS: A survey of the Utah population suggests that relatives of melanoma probands are at an increased risk of developing cancer of several other sites in addition to melanoma. Associations with pancreatic and eye cancers may be explained by the role of CDKN2A in these tumors. It is likely that other cancer predisposition genes exist to explain 60-80% of familial melanoma lacking a CDKN2A mutation. This data suggests that testing of other cancer predisposition genes may be warranted, especially in familial melanoma.

0457

Evaluation of baseline attitudes toward thorough skin self-examination (TSSE)

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Regular self-examination of the skin provides opportunities to detect melanoma at earlier stages and thus reduce skin cancer mortality. Current behavior change models suggest that attitudes are an important component of motivation and behavior. Attitudes toward skin thorough skin self-examination (TSSE) could influence motivation for or performance of TSSE tasks. The Check It Out project is a randomized trial of a multi-component intervention designed to encourage TSSE. Participants were recruited at the practices of primary care physicians. This analysis includes all participants who were subsequently randomized into the clinical trial (N=1356). All recruited participants completed a baseline survey, which included questions concerning attitudes about skin self-examination. This presentation examines the factor structure of participant responses to 25 attitude domain questions. Principal component analysis (with promax rotation) of attitude items revealed a 2 factor solution: positive domain attitudes toward TSSE (α=.82, factor loadings ranged 0.44-0.81) and negative domain attitudes toward TSSE (α=.77, factor loadings ranged 0.42-0.67). The factors accounted for 36% of the total item variance. The correlation between factors was not significant (r=-0.13). A measure of TSSE task performance was correlated with positive attitudes (r=+0.22, p<.0001) and with negative attitudes (r=-0.20, p<.0001). The attitude scales explained 7% of the variance in the TSSE measure. These results suggest that the attitude scales may be useful for predicting TSSE behavior.

0458

Integration of clinical and histological data by means of wireless PDA communication

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The purpose of this study was to develop a wireless database system that facilitates case-based entry of clinical and pathological data and promotes information/reporting accuracy. We designed database forms for Palm Pilot PDAs (personal digital assistants) for collection of pertinent clinical information relevant to dermatopathologic diagnosis. For the study, two dermatology residents and a dermatopathology attending physician were trained to use Palm Pilot PDAs with this database software. The clinical data generated from 45 randomly selected patients were wirelessly e-mailed to the dermatopathologist to aid histologic diagnosis. Following dermatopathology signout, the residents were sent both (1) digital reports and (2) conventional faxed paper reports. Diagnostic accuracy, reliability, and turnaround times of these two reporting methods were recorded and compared. In 39 of 45 cases, digital reports had better turnaround time than faxed paper reports; the other six cases coincided with periods during which the clinicians were absent and could not access digital reports. (In these instances, faxed paper reports were reviewed by a covering dermatologist). The average turnaround time improvement for digital reports was +51.2 hours (range -230.7 to +232.7 hours). Digital reports were also more reliable: in one case, a faxed paper report was physically lost, and never reviewed by the clinician. There were no electronic reports lost, because the clinician was alerted and sent another digital report if original report review was not acknowledged within a week. There was a 100% concordance rate between the data contained within the digital and faxed paper reports. In conclusion, our wireless database system design greatly improves turnaround time and delivery reliability over the conventional (faxed report) method. Our software is also easy to use (mean training time under 45 minutes). Our research, therefore, suggests that there is much potential for wireless communication in dermatology and pathology, specifically via PDAs and other wireless devices.

0459

Association between a promoter polymorphism in the *IL16* gene and contact allergy

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IL-16 is a natural ligand to the CD4 molecule that induces chemotactic responses in $CD4^+$ T cells, eosinophils and monocytes. More recently, IL-16 has been implicated in the regulation of Langerhans cell migration, and data from animal models suggest that IL-16 could play an important role during contact hypersensitivity reactions. Here, we investigated whether a promoter polymorphism in the *IL16* gene affects cytokine production and may influence susceptibility to contact allergy. PBMC were isolated from healthy donors (n = 36) and stimulated with LPS or LPS together with IL-10. Secreted levels of IL-16 were determined by ELISA. PBMC of carriers of at least one *IL16*-295*C allele (C at position -295) produced significantly more IL-16 in response to LPS/IL-10 than carriers of two wild-type alleles (T at position -295) (p = 0.0013). In a case-control study including healthy individuals (n = 310) and polysensitized individuals with eczema (confirmed contact sensitization to para-compounds and at least one other, structurally not related hapten; n = 86), the distribution of *IL16*-295 genotypes was significantly different in cases and controls (χ^2 = 12.79, p = 0.0021). In particular, individuals with an *IL16*-295*C/C homozygous genotype were significantly overrepresented among polysensitized compared with control subjects (7% vs. 1%; OR [95% CI] = 7.68 [1.88-31.37], p = 0.0043). We conclude that the *IL16*-295 promoter polymorphism may influence the production of IL-16 in response to IL-10, an anti-inflammatory cytokine released during contact allergic reactions, and may contribute to a genetic basis of contact allergy.

0460

Identification of mutations in the ARS gene encoding SLURP-1 in six Turkish families with mal de Meleda

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Mal de Meleda (MdM) is a rare form of palmoplantar keratoderma inherited in an autosomal recessive manner. It is characterized by erythema and hyperkeratosis of the palms and soles, extending to the dorsal aspects of the hands and feet (known as transgrediens keratoderma). Some of the commonly associated findings include nail abnormalities, keratotic plaques over the joints, perioral erythema, brachydactyly, and pseudoainhum. In 1998 the MdM gene was mapped to chromosome 8qter by linkage analysis. In a recent study, homozygous mutations in the ARS gene were identified in families with MdM, implicating ARS as the causative gene for this disease. Subsequently, a patient with MdM lacking mutations in ARS was reported, suggesting genetic heterogeneity. We describe six Turkish families with MdM in which three different homozygous mutations in the ARS gene were identified. While the R96X and 82delT are recurrent mutations, L98P is novel. Of interest, we identified the R96X mutation in four unrelated families we studied. The identification of recurrent mutations suggests that these may be hot spot mutations within the ARS gene, or alternatively a common ancestral allele. To determine whether the mutation in the Turkish population originated from a common ancestor, we analyzed the R96X allele signatures in these families. Our results showed that all four families shared a common haplotype at the MdM locus, suggesting a founder effect.

0461

Decision analysis as a tool to evaluate the utility of skin biopsy in acute graft-versus-host disease after allogeneic bone marrow transplant

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Controversy has surrounded the utility of skin biopsy in the diagnosis of acute graft-versus-host disease (GVHD) in allogeneic bone-marrow transplant (a-BMT) patients. Acute cutaneous GVHD manifests as a skin eruption seven to ten days post-transplant. A skin biopsy is frequently used as a screening tool to make the diagnosis. Often, skin biopsy findings cannot differentiate early acute GVHD from other causes of a skin eruption. Nonetheless, skin biopsies are being performed as confirmatory tests increasing the potential for morbidity and cost of care. This study employs decision analysis to address whether to perform a skin biopsy or not in a-BMT patients with a skin eruption up to 100 days post-BMT. First, a formal decision analysis has been performed, using the prior probabilities of disease (GVHD in a-BMT) and sensitivity and specificity of skin biopsy from data published. Pre-test probability of GVHD is 35-50% in post-BMT recipients, as obtained from numerous studies in the literature. A sensitivity analysis of prior prevalence of GVHD and test characteristics of skin biopsy has been performed. This will help inform physicians how decision-making changes based on differing opinions of prior-prevalence of disease, differing test characteristics and ways in which this model can be used at various institutions. Second, a survey of dermatologists and oncologists who frequently treat GVHD patients has been performed nationally. Individual physician estimates for prior probability of GVHD and sensitivity and specificity of skin biopsy will be used to analyze their own decision making based on clinical scenarios presented in the survey. Overall, this is a novel approach to study decision making in dermatology. Specifically, this preliminary work will form the basis for further investigation to delineate where improvements can be made in our approach to the diagnosis and management of acute GVHD.

0462

Providing dermatological care to the indigent population

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Medicaid is the government-subsidized program for the indigent and disabled population in the United States, and funds 11.6% of the population. Moreover, an additional 14.6% of the population in the U.S. is uninsured. Medicaid and indigent care patients pose difficult challenges in dermatology clinics, including poor reimbursement, restricted formularies, and the complexity of medical care. To date, there have been no published manuscripts discussing indigent care and dermatology in the U.S. We have determined indigent care providers of dermatological services in Detroit, Michigan, and the United States. We surveyed all Michigan dermatologists to determine who provides care for Medicaid and indigent care services, and received responses from 145 of 249 dermatologists (58%). Currently, 25.5% of dermatologists see no Medicaid patients and an additional 44.1% see <1%; fewer dermatologists see these patients now compared to 5 years ago. University Dermatology, the faculty practice of Wayne State University, treats the vast majority of this patient cohort, with Medicaid patients accounting for 40% of the clientele. To determine indigent caregivers nationally, a similar survey was sent to all members of the Association of Professors of Dermatology, and 52/100 responses were received. 78% of programs have state, city, or county hospital-based programs to absorb the costs of patient care and 42% of programs receive funding to cover faculty salaries. Of the 10 largest metropolitans, 3 do not have a state, city, or county-based indigent care program (Detroit, Washington, D.C., and Philadelphia). Indigent care is dispersed among several programs in Washington, D.C., and Philadelphia, but University Dermatology is the principle caregiver in metropolitan Detroit. Of the faculty practices, over 27% see <1% indigent patients and 69% see <5%. In sum, few dermatologists provide substantial indigent care, both in private practice settings and in academic medicine. Many challenges lie ahead in order to properly care for this patient cohort.

0463

Utilization of resources in a practice that provide care to private pay and indigent patients

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Medicaid and indigent care patients pose difficult challenges in dermatology clinics, including poor reimbursement, restricted formularies, and the complexity of medical care. To date, there has been a paucity of investigations regarding the care of these patients. We have examined the utilization of resources by Medicaid and indigent care patients in an office-based dermatology practice that cares for private pay and indigent patients. Specifically, we have determined no-show rates, incoming calls, outgoing calls, and physician calls at our practice site, in which state-funded programs cover 40% of patient visits. During a five-month period, 6594 appointments were analyzed, and the average no-show rate was 52.4% for all patients. Medicaid patients had a 57% overall no-show rate, in contrast to non-Medicaid patients who had a 48% no-show rate. A total of 537 incoming calls were surveyed, with 55% from Medicaid patients. The majority of incoming calls were for appointments, with a disproportionate number of new appointments for Medicaid patients. A total of 397 outgoing calls were surveyed, with Medicaid patients accounting for 55% of all calls. Most calls, 68%, were made to confirm appointments. A total of 42 physician calls were surveyed, and 79% of these calls were made to Medicaid patients. For incoming, outgoing, and physician calls, those to and from Medicaid patients were longer, and accounted for the majority of calls regarding medication issues and referrals. Medicaid patients utilize the majority of personnel resources, thereby increasing overhead costs. Therefore, office-based practices that see a large proportion of Medicaid patients are at a distinct disadvantage.

0464

Quality of life and psychological distress in older patients with psoriasis

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The ageing population is a growing public health concern. Our aim was to describe patterns and levels of quality of life (QoL) and psychological distress in patients with psoriasis aged 60 years or more. All adults hospitalised at IDI-IRCCS from Feb. 2000 to Feb. 2002 with psoriasis who gave their informed consent were given the Skindex-29 QoL questionnaire and the 12-item General Health Questionnaire (GHQ-12). Skindex-29 consists of three scales assessing social functioning, emotional state, and symptoms. The GHQ-12 measures psychological distress. In both questionnaires higher scores indicate a worst condition. Clinical severity was measured using PASI and SAPASI. Comparisons between groups were made using the Mann-Whitney U test. We studied 936 patients. Of these, 223 were 60y old. PASI and SAPASI were similar in older and younger (age <60y) patients, but QoL was significantly more impaired in the older group for all Skindex-29 scales (all p-values<0.01). Also GHQ-12 scores were significantly higher (p=0.013). Among the older patients, women had much higher Skindex-29 and GHQ-12 scores, with increases ranging from 28% for the emotions to 47% for the social functioning scales, all p-values<0.001, while clinical severity did not differ in the two genders. Duration of disease (cutoff 5 years) did not change scores significantly, although patients with longer experience of disease had higher scores on the emotions and social functioning scales. In conclusion, we observed a strong association between older age and poor QoL and increased psychological distress. Women seemed to suffer a heavier burden of disease. These observations should alert dermatologists that similar levels of "objective" measures of clinical severity may be associated with significantly different levels of QoL and psychological distress in patients with psoriasis. Particular attention should be devoted to older patients, and among these, to women.

0465

Dermatological care in Chile

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The aim of the study is to assess the quality of dermatological care in Chile. A Chilean Database of all dermatologists in the country was consulted, and a rate was calculated using the Chilean Census data. There are 300 dermatologists in Chile for a population of 15 million people, that is a rate of 1 dermatologist per 50,000 people. Two thirds of the dermatologists are based in Santiago (Metropolitan area) in which there is a third of the population, with a rate of 1 dermatologist per 25,000 people. In some regions in Chile there is an enormous shortage of specialists. The rate of dermatologists per population is less than the rate in the US and more than the rate in the UK. An increase in specialists is required in Chile particularly in rural areas and in some extreme regions in the country.

0466

Differences in quality of life of patients with nonmelanoma skin cancer at private and Veterans Affairs clinics

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We have previously reported that patients with nonmelanoma skin cancer treated in a private setting were more likely than those treated at a VA clinic to be treated with Mohs surgery, even after controlling for demographic and clinical features that might have affected treatment choice. The goal of this study is to learn how patients at the VA and private sites differed in the effects of their tumors on their quality of life before therapy. We performed a prospective cohort study of consecutive patients with non-recurrent nonmelanoma skin cancer diagnosed over 2 years at two sites, a private faculty practice (PRIVATE, 837 patients) and the dermatology clinic at the affiliated VA hospital (VA, 540 patients). Data were collected from medical records and patient reports using standardized dataforms. Skin-related quality of life was measured with Skindex-16; results are reported as 3 subscales: Symptoms, and the effects of skin disease on Emotions, and social and physical Functioning. Scores are reported as values between 0 and 100; higher scores indicate worse quality of life. The response rate was 60%. Compared with patients at the PRIVATE site, the VA patients reported worse symptoms from the skin cancer (25 vs 18, p<0.01), and more effect of the skin cancer on their functioning (16 vs. 12, p=0.04), but not on their emotions (40 vs 41, p=0.64). VA patients were less likely than PRIVATE patients, however, to be worried about treatment (36 vs 42, p=0.01) or to be concerned about scarring (24 vs. 32, p<0.01). Finally, compared to PRIVATE patients, VA patients reported that the condition of their skin was less important to their overall quality of life (36% very or extremely important, compared to 48%, p<0.01). We conclude that although nonmelanoma skin cancers affected the quality of life of patients at the VA more than at the PRIVATE site, patients at the PRIVATE site were more worried about treatments and scarring. This difference may have affected the choice of treatments by clinicians at the two sites.

0467

Transcription analysis of human peptidylarginine deiminase type I and type III genes

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Peptidylarginine deiminase (PAD) catalyzes the conversion of arginine residues to citrulline residues in the presence of calcium ion. At least four isoforms types have been identified. The type II and IV are widely expressed in various tissues. The type I is detected only in the epidermis and uterus. The type III is present only in the hair follicle and epidermis. The gene of type III closely links to the type I on human chromosome 1. In order to further define PAD regulation and function, we have isolated and characterized the promoter of the structural genes of type I and III. Screening of human genomic libraries and combining to PCR with human genomic DNA resulted in the isolation of 2.8 kb type I and 2.4 kb type III sequence upstream the translation start site. Deletion analysis in normal human epidermal keratinocytes (NHEKs), HaCat, COS-7 and HeLa cells identified a critical region for type I promoter activity between -106 and -156. -94/-276 region was required for normal activity for type III. Analysis of binding sites deletion imply that myeloid zinc finger 1 (MZF1) and stress-response element (STRE) are critical cis-element for type I promoter activity, but CCAAT-box and SP1 cis-element are required for type III. The time-course with transfer promoter-luciferase vector of type I and III to different subculture cells of NHEK also revealed that regulation patterns were different between type I and III. Our results indicate that the transcription of PAD type I and III are regulated by different factors, although both of them are expressed in the skin.

0468

Cystatin M/E deficiency in mice causes faulty cornification and impaired barrier function

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Cystatin M/E (CST6), a new member of the cystatin gene family, has a restricted expression pattern in humans, which is largely limited to cutaneous epithelia. Although cystatin M/E possesses two distinct biochemical properties, being a cysteine proteinase inhibitor and a substrate for transglutaminase, its physiological function is unknown. We isolated and characterised the mouse Cst6 orthologue and mapped the gene to the proximal end of mouse chromosome 19. This region corresponds to the locus of the spontaneous harlequin ichthyosis (ichq) mouse mutation, for which no causative gene has been identified sofar. We found a nonsense mutation in the Cst6 gene of BALB/cJ-ichq/+ mice, which precludes the synthesis of functional protein. Immunohistochemistry confirmed the absence of cystatin M/E at the protein level in phenotypically mutant mice. Mice that are homozygous for two null alleles display a hyperplastic, hyperkeratotic epidermis and abnormal hair follicles, and die between 5 and 12 days of age. As skin is the main site of cystatin M/E expression we surmise that disturbed epidermal homeostasis is a probable cause of death in these mutant mice. From day 5 onwards, homozygous mutant mice have decreased body weights compared to controls. In addition, we demonstrated highly increased levels of transepidermal water loss in phenotypically mutant mice, which make it very likely that these mice die of dehydration. In wild-type mice, cystatin M/E was found in the stratum granulosum and in the infundibulum of the hair follicle indicating that the anatomical site in the skin where cystatin M/E is normally expressed correlates with the abnormalities at the tissue level in mutant mice. Our data provide evidence that cystatin M/E is essential for correct formation of cornified layers, intact barrier function, and viability.

0469

In vivo fluorescence confocal microscopy of skin in the transgenic green fluorescent protein mouse

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In this study we describe the microscopic architecture of transgenic green fluorescent protein (GFP) mouse skin, as viewed in vivo using a hand held fluorescence confocal microscope. Additionally, the in vivo morphology of an induced inflammatory response imaged with the same system is presented. Transgenic GFP mouse (C57BL/6-TgN (ACTbEGFP)10sb) skin (ear and abdomen) was imaged in vivo with a miniaturized fluorescence confocal microscope system (Stratum, Optiscan Pty Ltd). GFP was excited with 488nm light and fluorescent emission was detected at >505nm. The imaged sites were biopsied and cryosectioned. The in vivo images were correlated with ex vivo fluorescence microscopy and conventional histology. An immune modulator (imiquimod 5%), was topically applied to the ear and abdomen for 24 hours with occlusion to induce an inflammatory response. After treatment, the skin sites were imaged with the in vivo confocal system and again biopsied for cryosectioning. The in vivo images were correlated with ex vivo fluorescence microscopy, conventional histology, and immunohistochemistry utilizing CD11c antibody to identify dendritic cells. On in vivo confocal imaging, keratinocytes, hair follicles, sebaceous glands and blood vessels displayed bright fluorescent emission, allowing them to be clearly viewed. In addition, fat and chondrocytes in the subcutaneous layer of the ear were observed to display a strong fluorescent signal. In vivo confocal imaging of imiquimod treated skin showed a new population of cells, some with dendritic morphology, in the deep epidermis and dermis. Conventional histology confirmed a population of inflammatory cells in the dermis, and CD11c antibody immunostain confirmed the presence of mouse dendritic cells. Real-time imaging of transgenic GFP mouse skin with a handheld fluorescence confocal microscope provides a unique model for the study of mouse skin in vivo. Furthermore, the ability to monitor a dynamic process in vivo has been demonstrated.

0470

Phospholipase B expression in human epidermis

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Phospholipase B (PLB) is an enzyme that can remove both sn-1 and sn-2 fatty acids of glycerophospholipids and thus displays phospholipase and lysophospholipase activities. Analysis of human epidermis homogenates indicated the presence of a 97 kDa PLB protein, being enriched in the soluble fraction. Immunolabelling and in situ hybridization experiments showed that this enzyme is expressed in the different layers of epidermis with an accumulation at the dermo-epidermis junction. RT-PCR data indicated that PLB is specifically expressed in natural and reconstructed epidermis and not in keratinocytes in culture suggesting a role in differentiation process. Using 3-RACE-PCR and screening of human genome databases, we obtained a 3600 bp cDNA coding for human PLB that share 74% identity with guinea pig PLB. Altogether, our results provide the first characterization of human homologue of PLB. This enzyme should play an important role in the generation of free fatty acids involved in the epidermal barrier function.

0471

***Hoxb13* knockout adult skin displays fetal-like characteristics and enhanced wound healing**

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The purpose of this study was to determine if eliminating Hoxb13 function in adult skin would enhance wound healing. Compared to adult skin wounds, fetal skin wounds made early in gestation heal faster and with no scar. Two important factors implicated in fetal scarless wound healing are the degree of differentiation and the higher levels of hyaluronan (HA) present in fetal skin and wounds. HA is thought to inhibit differentiation. Hoxb13, a member of the highly conserved family of Hox transcription factors, is expressed in fetal and adult skin. Its expression is significantly downregulated in fetal wounds that heal without a scar compared to adult wounds. We postulated that eliminating Hoxb13 from adult skin could improve wound repair. To test this, we evaluated adult skin wounds in a *Hoxb13* knockout (KO) mouse line. Tensiometry was used to measure the breaking strength of incisional wounds. Overall, *Hoxb13* KO wounds were stronger than wild-type (WT). Histological evaluation indicated that *Hoxb13* KO incisional wounds healed faster and with less scar. *Hoxb13* KO excisional wounds closed at a faster rate compared to WT. We determined that *Hoxb13* KO adult skin has higher levels of HA and reduced expression of several differentiation markers as shown by biochemical and microarray analyses, respectively. We conclude that one function of Hoxb13 in adult skin is to promote differentiation. We further propose that loss of Hoxb13 function in skin results in a more fetal-like state, and one consequence of this is improved wound healing.

0472

Phenotypical characterization of transit amplifying cells in normal human epidermis

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The epidermis contains two types of proliferative keratinocytes: stem cells with unlimited self-renewal capacity and their daughters, transit amplifying cells, which have a more restricted proliferative potential and high probability of undergoing terminal differentiation. The identification and isolation of stem cells and their progeny is critical to our understanding of their growth regulation during homeostasis, wound healing and disease. Using fluorescence-activated cell sorting (FACS), immunohistochemistry and in vitro studies, we aspired a more precise isolation and characterisation of transit amplifying cells, which could clarify their role in hyperproliferative skin disorders. Comparing stem cell and transit amplifying cell-enriched fractions derived from normal skin biopsies, we addressed the question whether differences in growth behaviour and expression patterns could be demonstrated. Keratinocyte fractions were FACS enriched for stem or transit amplifying cells on the basis of β1- integrin expression and cultured on cover slips to allow characterisation of individual colonies. Subsequently, immunohistochemistry was used to examine the expression pattern of cell surface markers (β1-, α6- integrin, transferrin receptor), keratinocyte differentiation (cytokeratin 10 and 6) and proliferation markers (Ki-67 antigen). Culturing keratinocytes on cover slips proved beneficial with respect to morphological characterisation of individual colonies, and suggested differential growth behaviour of colonies from β1-integrin bright and dim keratinocytes derived from normal skin. Furthermore, remarkable differences were found with respect to expression patterns of cytokeratin 10 and 6 in stem cell or transit amplifying cell enriched fractions. We propose that these findings can be of relevance for further studies on transit amplifying cells and their role in hyperproliferative skin disorders.

0473

Role of AHNAK/desmoyokin in skin development and function

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Desmoyokin was first isolated from bovine muzzle epidermis and thought to be an epidermal desmosome related protein. We previously demonstrated that desmoyokin gene is identical to AHNAK gene which is expressed ubiquitously and down-regulated in neuroblastomas. According to various in vitro experimental studies, AHNAK/desmoyokin was expected to be associated in epidermal cell adhesion, signal transduction pathway of cell proliferation and differentiation, tumorigenesis, and embryonic development, but no evidence have been revealed by means of in vivo experiments. In order to investigate the functional roles of AHNAK/desmoyokin in vivo, we generated a null mutation in mice. Southern analysis and immunoprecipitation analysis indicated that AHNAK/desmoyokin was successfully disrupted. Mutant mice were viable and fertile and showed no gross developmental abnormalities. Integrity of the skin appeared normal in mutant mice. Electron micrographs of skin sections revealed that the ultrastructure of cell-cell junction including epidermal desmosomes in mutant mice were indistinguishable from that of wild-type animals. No tumorigenesis has been observed in mutant mice up to 12 months of age. Therefore, we concluded that AHNAK/desmoyokin is not essential for epidermal cell adhesion, and that AHNAK/desmoyokin deficiency has minimal effect against cellular proliferation and differentiation process, tumorigenesis, and developemental process in vivo. Recently, AHNAK/desmoyokin was shown to be immunoprecipitated with monoclonal antibody specific to a 3.7 million huge protein named of titin which shares structural similarities with AHNAK/desmoyokin. That report and our results supposed that titin might largely compensate for the lack of AHNAK/desmoyokin.

0474

Adipogenic hormones differentially regulate lipogenesis in human sebocytes and keratinocytes

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An understanding of the factors that regulate sebaceous gland lipogenesis is key to the discovery of drugs designed to reduce sebum production and improve acne. The adipogenic hormones methylisobutylxanthine, dexamethasone and insulin (MDI) are known to promote the differentiation of preadipocytes into adipocytes, a process that is accompanied by an increase in cell volume and lipid synthesis. Adipocyte differentiation in turn is accompanied by an increase in the expression of the peroxisome proliferator-activated receptor gamma (PPARg). The PPARs are expressed in human skin and sebaceous glands. The goal of this study is to test the hypothesis that adipogenic hormones induce lipogenesis in human sebocytes and that this process is accompanied by changes in the expression of PPARs. SV40-transfected human sebocytes (SEB-1 cells) were treated with MDI medium or basal medium for 3 days, followed by analysis of 14C-acetate incorporation into neutral lipids and uptake of 14C-oleic acid from the medium. Parallel experiments were performed in a spontaneously immortalized human keratinocyte line (HaCaT). Treatment of cells with MDI resulted in a significant increase in the incorporation of acetate into neutral lipids in SEB-1 sebocytes compared to HaCaT keratinocytes. When compared to basal medium, MDI significantly increased the incorporation of acetate into cholesterol, triglycerides, and cholesterol esters in both SEB-1 cells and HaCaT cells. In SEB-1 cells, but not HaCaT keratinocytes, MDI treatment significantly increased the incorporation of acetate into wax esters, a lipid class characteristic of sebocytes. MDI treatment significantly increased the uptake of fatty acids by SEB-1 cells compared to basal medium. These data further substantiate the hypothesis that sebocytes and adipocytes share common regulatory influences whereas sebocytes and keratinocytes respond differently to adipogenic stimuli. Further elucidation of the mechanisms regulating sebaceous gland differentiation may provide a rationale for the role of PPAR ligands in this process.

0475

pH directly regulates epidermal permeability barrier homeostasis and stratum corneum integrity/cohesion

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Both exposure of stratum corneum (SC) to neutral pH buffers and blockade of acidification mechanisms, disturb cutaneous permeability barrier homeostasis and SC integrity/cohesion, but these approaches all introduce potentially confounding variables. To study the consequences of SC neutralization, independent of hydration, we applied two chemically-unrelated superbases, 1,1,3,3-tetramethylguanidine (TMG) or 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU), and assessed whether discrete pH changes alone regulate cutaneous permeability barrier function and SC integrity/cohesion, as well as the responsible mechanisms. Both TMG and DBU applications increased skin surface pH in parallel with abnormalities in both barrier homeostasis and SC integrity/cohesion. The latter was attributable to rapid activation (< 20 min) of serine proteases (SP), assessed by in situ zymography, followed by SP-mediated degradation of corneodesmosomes (CD). Western blotting revealed degradation of desmoglein 1, a key CD structural protein, in parallel with loss of CD. Co-application of SP inhibitors with the superbase prevented the abnormality in SC integrity /cohesion. The superbases also delayed permeability barrier recovery, attributable to decreased -glucocerebrosidase activity, assessed zymographically, resulting in a lipid processing defect on electron microscopy. These studies demonstrate unequivocally that SC neutralization alone provokes SC functional abnormalities, including aberrant permeability barrier homeostasis and decreased SC integrity/cohesion, as well as the mechanisms responsible for these abnormalities.

0476

A sustained increase in stratum corneum pH alone alters SC function

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The acidic nature of mammalian stratum corneum (SC) has been appreciated for more than a century. Recent data, based on mouse models of SC alkalization (exposure to alkaline buffers and acidification pathways inhibitors), suggest that an acute increase in SC pH provokes alteration in skin barrier homeostasis and SC integrity/cohesion. To assess the functional outcomes of prolonged SC neutralization on epidermal barrier homeostasis we applied a superbase, 1,1,3,3-tetramethylguanidine (TMG) twice daily for 5 consecutive days. Using this approach, we assessed whether pH changes alone alter cutaneous permeability barrier function and SC integrity/cohesion, as well as the metabolic basis for such alterations. A sustained neutralization of SC pH was paralleled by abnormalities in both barrier homeostasis and SC integrity/cohesion attributable to an increase in serine protease (SP) activation visualized by in situ zymography. Quantitative electron microscopy and western blot analysis demonstrate a decrease in CD density and degradation of desmoglein 1 (DSG1), a key constituent of CDs, respectively. Also, superbase daily application induced alterations in permeability barrier homeostasis paralleled by a decrease in beta-glucocerebrosidase (beta-GlcCerase) activity due to a decrease in enzyme mass. Co-application of STI prevented the decrease in beta-GlcCerase mass and the abnormality in barrier function. Finally, the activation/release of primary cytokines, TNF-alpha, IL-1alpha and beta and IL-1ra, observed following SC sustainable neutralization was successfully overridden by STI treatment. In conclusion, sustained SC neutralization alone can provoke functional abnormalities mediated by the activation of SP, including decreased SC integrity/cohesion, impaired permeability barrier homeostasis, and the activation/release of cytokines.

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Unsaturated fatty acids cause parakeratosis by inducing cornification in the middle layer of epidermis

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Unsaturated fatty acids, such as oleic acid, in sebum are known to be irritative and are considered to be one of the causes of comedo. Our observations suggested that unsaturated fatty acids might also be a cause of noticeable facial pores. Many parakeratotic corneocytes were detected around facial pores, and topical application of unsaturated fatty acids to human or mouse skin induced parakeratosis. In order to clarify the mechanism of induction of parakeratosis by unsaturated fatty acids, we applied oleic acid to hairless mouse back skin, and differentiation markers were analyzed histologically. Cornified envelope formation was examined using an in situ transglutaminase (TGase) activity assay. The activity was detected in the middle layer of epidermis of oleic acid-treated skin, whereas it was restricted to the granular layer in normal skin. Localization of the enzyme in the middle layer of the epidermis was also confirmed immunohistologically. On the other hand, TUNEL-positive cells were observed only in the granular layer in both oleic acid-treated skin and normal skin. This indicated that loss of nuclei in terminal differentiation of keratinocytes was restricted to the granular layer even in thickened epidermis treated with oleic acid. These results suggest that topical application of oleic acid induced formation of cornified envelope in the middle layer of the epidermis without loss of nuclei, and this led to parakeratosis.

0478

The metabolism of phytosphingosine in human keratinocytes

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Natural ceramides have various sphingoid bases. Mammals take in these ceramides, not only those with a sphingosine base but also those with a phytosphingosine base, in their diet. However, most mammalian organs have ceramides only with a sphingosine base but not with a phytosphingosine base. As an exception, ceramides in the epidermis have three types of sphingoid base, sphingosine, phytosphingosine, and dihydrosphingosine. Although all these types of ceramides can be converted to sphingoid by epidermal ceramidase, the sphingoid in the stratum corneum is mainly composed of sphingosine and contains very little phytosphingosine. Although much is known about sphingolipid metabolism, little is known about the metabolism of phytosphingosine in mammals. In this study, we have examined the metabolism of sphingolipids to clarify whether human keratinocytes can metabolize phytosphingosine. Cultured human keratinocytes take up most of the applied phytosphingosine within 1 hour as well as they do sphingosine. Within 24 hours, most of that phytosphingosine disappears, and less than half of the sphingosine remains in the cells as the free form. Surprisingly, an increase in free sphingosine or sphinganin in keratinocytes was observed following the uptake of phytosphingosine. From these results, human keratinocytes appear to take up phytosphingosine actively and convert at least part of that into sphingosine. Analysis of sphingolipids in the keratinocytes revealed an increase in ceramide and glucoslyceramide with the sphingosine base following incubation with sphingosine. In contrast, incubation with phytosphingosine increases the ceramides and glucosylceramides with the phytosphingosine base more than with the sphingosine base. These conversions were observed with a peak around 1 hour after application. These data suggest that keratinocytes take up phytosphingosine actively and metabolize that to other sphingolipids relatively quickly.

0479

C1orf10, a novel marker of late terminal keratinocyte differentiation

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The epidermal differentiation complex (EDC) on human chromosome region 1q21 contains three clustered families of genes encoding: (a) precursor proteins of the cornified cell envelope (involucrin, loricrin, SPRRs, xp5/LEP proteins) characterized by short tandem peptide repeats, (b) S100 proteins containing Ca^{2+}-binding EF-hands and (c) the fused gene family (profilaggrin, trichohyalin, repetin, hornerin) combining both characteristics. Recently, a novel gene C1orf10 with esophageal expression has been reported but its genomic and structural characteristics as a fused gene of the EDC were not recognized. The C1orf10 gene has 3 exons, the first untranslated, and a protein product of 495 aa with a N-terminal Ca^{2+}-binding motif of 90 aa and two consecutive repeats sequences of 60 aa. Here we identified expression of C1orf10 transcripts in fetal bladder, scalp, foreskin and in cultured keratinocytes by RT-PCR analysis. C1orf10 specific antibodies were raised against synthetic peptides in rabbits and affinity purified. SDS-PAGE and Western blot analysis revealed a protein of 57 kDa in the cytoskeletal fraction of sequentially extracted foreskin. Immunofluorescence microscopy revealed a punctate staining in the granular layer of the epidermis and in mucosal epithelia. To investigate the subcellular localization and to understand the function of the C1orf10 protein and its domains, different GFP fusion constructs were generated in the mammalian expression vector pEGFP. HeLa cells transfected with these constructs showed a diffuse cytoplasmic distribution of the protein except for C1orf10 ΔEF which exhibited a granular pattern while EF-hand alone localised similarly to that of GFP control. This suggests that N-terminus of C1orf10 differs from the function of the profilaggrin B domain which localises primarily to the nuclear compartment. This is consistent with the absence of a known nuclear localisation signal in C1orf10. In conclusion, C1orf10 is a novel marker of late epidermal differentiation and mainly located in the cytoplasm of granular cells in the epidermis and mucosal epithelia.

0480

Lamellar granule proteins are sequentially synthesized and transported through the trans-Golgi network in keratinocytes

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Lamellar granules (LGs) are small, membrane-bound organelles of the epidermis. Several reports have suggested that LGs originate from Golgi apparatus. LGs contain lipids and several proteins including corneodesmosin (Cdsn), kallikrein (KLK) 7, KLK8 and elafin. Differences in the timing and position of LG protein synthesis, and whether these are sorted into different granules or are transported into a single type of granule remain unknown. To address these questions, we studied LG protein localization in human epidermis using specific antibodies. Confocal laser scanning microscopy revealed that these proteins were co-localized with TGN46, a glycoprotein found primarily in the trans-Golgi network (TGN). Cdsn, KLK7 and KLK8 were all expressed in the superficial epidermis of normal and psoriatic skin. Elafin was not expressed in normal epidermis, but was expressed in psoriatic epidermis. In normal skin, Cdsn was the first to be expressed, before KLK7 and KLK8, whereas in psoriatic skin elafin was the first one. Immunoelectron microscopy revealed that LGs were part of the TGN. There was a definite but incomplete compartmentalization of each protein in the TGN; fusion of some compartments and even close association of different molecules were observed, indicating that different LG proteins share a trafficking system. The present study provided strong evidence for the sequential synthesis and trafficking of various LG proteins through TGN and heterogeneity in LG contents.

0481

Three-dimensional analysis of human epidermal structure from *in vivo* confocal microscopic images

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In vivo confocal laser scanning microscopy (CLSM) has made it possible to observe non-invasively the inside of living human skin, mainly epidermal layers. We have developed a method to reconstruct the three-dimensional (3D) structure of the epidermis from a sequence of automatically obtained confocal images. A new image analysis method of 3D edge detection and 3D reconstruction based on the Snakes method was used. The Snakes method[1)] is to evolve a curve, which consists of initial broadly positioned control points on the image, through an image-derived energy-minimization process so that it ultimately conforms to the feature of interest, such as the dermo-epidermal junction. To analyze the volumetric confocal images, we have developed the 3D Snakes method, which uses the same image-derived energy functions as the Snakes model, but deforms them into 3D space. This new method makes it possible to detect the area of epidermis automatically and to visualize the precise structure of the epidermis, which consists of the surface of the stratum corneum and the dermo-epidermal junction. This 3D reconstruction of the epidermal structure allows us to analyze the thickness of the epidermis and the undulation of the dermo-epidermal junction in detail. Our study of the structural variations of the epidermis in normal skin with this method revealed substantial differences between subjects of different ages, and between males and females, not only visually, but also quantitatively. Furthermore, detection of melanins, which appear as characteristically bright reflective clusters in the confocal images, was investigated to elucidate the 3D distribution of melanins within the epidermis. We conclude that *in vivo* CLSM will be a very useful tool for the precise 3D analysis of epidermal structure.

0482

Unsaturated fatty acids in sebum cause parakeratosis around facial pores

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Noticeable pores are one of the facial characteristics that concern women of various ages. Because little is known about such facial pores, the physiological characteristics of the cheek skin of healthy women volunteers were investigated. The volunteers were classified into three groups according to apparent facial pore size. The group with larger pores was found to have more parakeratotic corneocytes and higher transepidermal water loss (TEWL) values. This suggests that women with large facial pores may have immature corneocytes. The group with larger pores was also found to have larger amount of sebum, especially unsaturated free fatty acids such as palmitoleic acid (16:1) and oleic acid (18:1). Corneocytes were obtained from human facial skin by a tape-stripping method and nucleosomes were stained with Hoechst 33342. Parakeratotic corneocytes were mostly found around sebaceous ducts which had the appearance of cone-shaped hollows containing sebum. We applied sebum components to mouse skin, and found that unsaturated fatty acids induced significant parakeratosis, whereas other components, such as saturated fatty acids, triolein and squalene, had little effects. These results suggested that unsaturated fatty acids in sebum cause parakeratosis around sebaceous ducts, and this might lead to noticeable facial pores.

0483

Melanocortin 5-receptor: a marker of human sebocyte differentiation *in vitro*

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Evidence for Melanocortin 5-Receptor (MC5-R) expression in human sebaceous glands and cultures has been reported by Thiboutot et al., (2000), while studies by Bohm et al., (2002) failed to show MC5-R expression in immortalized SZ 95 sebocytes. Since MC5-R has been shown to play a role in sebum regulation in mice (Chen et al, 1997) we examined human sebocytes in primary cultures and analyzed the correlation between sebaceous cell maturation, resulting lipid granule formation, and MC5-R expression. Sebocytes derived from human facial skin were induced to differentiation by melanocyte stimulating hormone (MSH) or cholera toxin. Melanocortin 5-R expression was detected by immunostaining in cells 24-48 hours prior to the appearance of lipid droplets in their cytoplasm and remained expressed in differentiated sebocytes, fully lipid laden. The immunoreactivity was completely removed by absorption with the immunizing peptide. In addition, using RT-PCR, presence of MC5-R was documented in differentiating sebocytes, but not in non-induced cultures. To establish whether a correlation between visual observation of lipid granules and sebaceous lipid production exists, a comparative examination of neutral lipids in cultured cells was performed. Analysis of 14C labeled lipids by high performance thin layer chromatography showed: 1) squalene and wax esters to be detectable only in differentiated cultures; 2) an increase in sebaceous neutral lipids (cholesteryl esters, and triglycerides) in differentiated versus undifferentiated cells. Our studies show that sebocytes in culture can be induced to differentiate into sebaceous lipid producing cells, displaying prominent lipid granules in their cytoplasm. The data also suggest that MC5-R can be detected both in early and late stages of sebocyte differentiation. Since MSH, the agonist for MC5-R can induce sebocyte differentiation, our results suggest involvement of MC5-R in human sebogenesis.

0484

Epidermal dopamine receptors contribute to skin barrier homeostasis

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Dopamine is a neurotransmitter and five subtypes of G protein-coupled receptors execute dopaminergical action in nerve cells. Especially, the dopamine receptors D2 (DRD2), D3 (DRD3) and D4 (DRD4) play important roles in neuronal activity by down-regulating the intracellular cAMP signaling pathway. In the present study, we demonstrated the expression of dopamine receptors in the epidermis and their functional contribution for epidermal barrier homeostasis. The immunohistochemical study showed the localization of DRD2 in the basal layer and that of DRD4 in stratum granulosum of epidermis of both human and hairless mouse. RT-PCR also revealed the transcription of DRD2 and DRD4 genes in cultured human keratinocytes and hairless mouse epidermis. In hairless mice, topical application of Bromocriptine, known as a common agonist for both DRD2 and DRD4, accelerated the epidermal barrier recovery after barrier disruption by tape stripping and it also reduced epidermal hyperplasia induced by acetone treatment under a low environmental humidity. On the other hand, topical application of the DRD2-specific antagonist L-741,626, delayed barrier recovery and enhanced epidermal hyperplasia. These results suggest that epidermal DRD2 and/or DRD4 is associated with epidermal barrier homeostasis.

0485

Flotillas of lipid rafts in transit amplifying cell-like keratinocytes

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Lipid rafts are dynamic membrane microdomains enriched in cholesterol and sphingolipid. Since many membrane proteins (e.g. growth factor receptors) partition into rafts, these structures play a crucial role in the regulation of a variety of cellular processes, such as proliferation, apoptosis and cell motility. We have previously described that large lipid raft aggregates are readily detectable on cultured keratinocyte cell line HaCaT by staining with the fluorescein-tagged cholera toxin (CTx-FITC). In this paper we adopted this method for the detection of lipid rafts in human epidermis and keratinocytes in culture. The double labelling with CTx-FITC and CD29 (β1 integrin) antibody showed the non-overlapping clusters of basal cells: $CD29^{dim}CTx\text{-}FITC^{bright}$ cells in the deep rete ridges and $CD29^{bright}CTx\text{-}FITC^{dim}$ cells at the tips of dermal papillae. A similar patchy, non-overlapping staining pattern was observed in vitro in confluent cultures of normal human keratinocytes and HaCaT cells, further confirming the inverse relationship between the expression of β1 integrin and the presence of lipid raft aggregates in the membranes. Studies on keratinocyte proliferation in vitro by means of labelling with bromodeoxyuridine and antibodies against cyclin E and Ki67 revealed that the $CTx\text{-}FITC^{bright}$ cells are mitotically active whereas a large proportion of $CTx\text{-}FITC^{dim}$ cells are quiescent. We conclude that the epidermal stem-like cells, previously shown to occupy the tips of dermal papillae and to exhibit high density of membrane β1 integrin have a low content of lipid rafts. In contrast, the putative transit amplifying cells in deep rete ridges show an enrichment in lipid rafts. Thus, lipid rafts may be a factor controlling the mitotic activity of epidermal keratinocytes and possibly the differentiation of stem cells into the transit amplifying cells.

0486

Influx of calcium and chloride ions into epidermal keratinocytes regulates skin water impermeable barrier homeostasis

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In the nervous system, influx of calcium and chloride ions into neurons regulates the signaling system by exitation and inhibition, respectively. In the present study, we demonstrated the effects of the ion influx into epidermal keratinocytes in the water impermeable barrier repair process of the skin after its damage. Topical application of the excitatory neurotransmitters, glutamate and nicotine, which activate the calcium channel, delayed the barrier repair after tape stripping. On the other hand, the inhibitory neurotransmitter, GABA and glycine, which activate the chloride channel, accelerated the barrier repair. Topical application of the calcium ionophore, ionomycine, delayed the barrier recovery and chloride ionophore 1 accelerated the barrier repair after the barrier disruption by tape stripping and acetone treatment. Ionomycine increased the intracellular calcium ion concentration in the cultuted keratinocyte while the chloride ionophore 1 increased the intracellular chloride ion concentration. Light microscopic and electro-microscopic observation showed the acceleration of the exocytosis of the lipid-containing lamellar body by the chloride ionophore 1 and the delay of the exocytosis by ionomycine. These results suggest that like the nervous system, influx of calcium and chloride ions into the epidermal keratinocytes plays a crucial role in cutaneous barrier homeostasis.

0487

The effect of increased gravitational forces on human keratinocytes

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Keratinocytes constitute the outermost layer of human skin. Many kinds of stresses including temperature, mechanical force, gravity, and osmotic pressure from the surroundings may affect keratinocyte growth and differentiation in vivo. Among those stresses, the effect of gravitational forces on keratinocytes has yet to be fully elucidated. The aim of this study is to examine the effects of gravitational force on human keratinocytes. A human keratinocyte cell line, HaCaT cells were cultured under standard conditions with or without pulsed application of centrifugal force. We used a simple tabletop centrifuge to apply a centrifugal force to HaCaT cells, and applied approximately 40G for 30 minutes every 24 hours. After 4 days, the pulsed centrifugal force induced a maximum 20% increase in proliferation as determined by the CellTiter 96(Promega, Madison, WI) assay. To determine the mechanism of this centrifugal force-induced keratinocyte proliferation, we conducted PCR-based, cDNA subtraction analysis. Total RNA was prepared from HaCaT cells after 4 days' culture with or without centrifugal force treatment. Full-length cDNAs were amplified using a PCR-based SMART&#65533;system (Clontech, Palo Alto, CA). Using these full-length cDNAs, subtractive hybridization was performed to determine the genes which were induced by centrifugal force using PCR-Select&#65533;cDNA subtraction kit (Clontech, Palo Alto, CA). A total of 300 subtracted cDNA clones were analyzed by differential hybridization, and several candidate genes with up regulated expression were identified.

0488

The cathelicidin antimicrobial peptide LL-37 induces proliferation of epithelial cells

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The antimicrobial peptide LL-37 is the C-terminal active fragment of the hCAP18, the single cathelicidin protein in human. We have recently shown that LL-37 is induced in association with wound healing and that it is lacking in chronic wound epithelium. The aim of the present study was to examine the potential role of LL-37 in the re-epithelialization process. We have used an ex-vivo wound healing model composed of organ cultured human skin in which re-epithelialization reproducibly occurs at 4-7 days. In this model LL-37 is strongly expressed in the epithelial cells migrating to cover the wound surface. Treating such wounds with LL-37 specific antiserum dose-dependently inhibited re-epithelialization and severely impaired the appearance of the individual keratinocytes. Evidence of cell proliferation assessed as immunoreactivity for Ki67 was lacking in the epithelium of the treated non-healing wounds suggesting that LL-37 may be involved in cell proliferation. Treating cultured HaCat cells and primary human keratinocytes with synthetic biologically active LL-37 peptide at 25, 50 and 100 µg/ml, dose-dependently increased proliferation rate of these cells. Cells were cultured at 2 and 10% fetal calf serum respectively. 100 µg/ml of LL-37 at 2% serum concentration severely affected the majority of cells indicating a cytotoxic effect. However, at 10% serum concentration no sign of cytotoxicity was detected, which confirms the observation that the cytotoxic activity of cathelicidin α-helical peptides, such as LL-37, is inhibited by the presence of serum. Proliferation was measured by flow cytometry and by assessing mitochondrial activity (WST-1). The increase in proliferation was 30-40 % higher than that of non-treated cells and equal or higher than that induced by epidermal growth factor. In summary, our data indicate a novel function for this peptide which in addition to being antimicrobial may have a growth-factor effect in epithelial cells.

0489

The characterization of epiplakin in NHEK cells

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Epiplakin, a member of plakin family, is a 450 kDa protein consisting of 13 repeats homologous to the B domain of desmoplakin. It lacks a rod domain and an amino-terminal domain characteristic of all other plakins. Epiplakin is expressed in various tissues such as liver, digestive tract, salivary gland, as well as skin. Here, we have characterized its distribution and the function in human primary epidermal keratinocytes (NHEK). A polyclonal antibody against the unique peptide sequences of the linker regions between the last 5 repeated domains was used as an epitope. The results from immnuofluorescence staining reveal that epiplakin is distributed in a filamenteus pattern in the cytoplasm with K5/K14 when cells are grown in low calcium medium (0.5 mM). Similar results were observed in HaCaT, A431 epidermal cell lines, but no staining was found in primary human fibroblasts. Interestingly, not all NHEK cells grown in low calcium medium showed positive epiplakin staining: those of a large, flat morphology showed the strongest staining. Also, the percentage of positive staining cells did not increase with cell density. However, the number of positive staining cells increased with time that NHEK cells were maintained in high calcium medium (1.2 mM). This was confirmed by western blots. In addition, western blots showed a very high molecular weight protein complex recognized by epiplakin antibody, suggesting epiplakin might become cross-linked with other epidermal proteins. In apparent support of a role in cell envelope barrier function, epiplakin co-localizes with K1/K10 filaments in 1-7 day high calcium cultures. Some epiplakin co-localizes with involucrin and transglutaminase 1 at the cell peripheral. It does not co-localize with profilaggrin granules. Taken together, these results suggest that (1) epiplakin is an important keratin filament-associated protein; (2) it might be an early differentiation marker; (3) its expression is calcium-dependent; (4) it interacts with different epidermal proteins during terminal differentiation; and (5) it is a potential precursor for the cell envelope.

0490

Beta-2-adrenergic receptor antagonist accelerates skin barrier recovery and reduces epidermal hyperplasia induced by barrier disruption

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Effects of topical application of adrenergic receptor agonists and antagonists on epidermal barrier repair rate after barrier disruption were studied. Agonists and antagonists of adrenergic beta 1 receptor did not affect the barrier repair rate. On the other hand, the adrenergic beta 2 receptor agonist, procaterol hydrochloride, delayed the barrier recovery and beta 2 receptor antagonist, ICI-118551, blocked the delay. Moreover, topical application of ICI-118551 alone accelerated the barrier recovery. Antagonists of alpha 1 and alpha 2 receptors did not affect the barrier recovery. The delay of barrier repair induced by prodaterol hydrochloride was blocked by a voltage-gated calcium channel blocker, verapamil. In cultured human keratinocytes, procaterol hydrochloride increased the intracellular calcium concentration and the increase was blocked by ICI-118551 and also by verapamil. Topical application of ICI-118551 partially blocked the epidermal hyperplasia induced by acetone treatment under low environmental humidity. These results suggest that the adrenergic beta 2 receptor is specifically associated with skin barrier homeostasis.

0491

Desmoglein 3: a novel negative marker for epidermal stem cells

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No single method has been universally adopted for identifying and isolating epidermal stem/ progenitor cells, and the emergence of new markers of stem cell populations is worth exploring. We recently showed that clusters of basal keratinocytes at the bottom of the rete ridges in human palm, previously recognized as a major repository of stem cells, had very low levels of desmoplakin protein and mRNA expression, compared with cells at the sides of the ridges or above the dermal papillae. Furthermore, in populations of palm keratinocytes, selected by rapid adhesion to type IV collagen, immunoblotting showed significantly reduced levels of desmoplakin and other major desmosome proteins. Here we report for the first time the use of desmoglein 3 (Dsg3), a major desmosomal cadherin, as a negative cell surface marker to select for epidermal stem/progenitor cells from adult human palm skin. Using FACS analysis we have been able to identify a cell population with a low Dsg3 expression which is also characterized by a high clonogenecity, colony forming efficiency and proliferative potential but with a low ability to form paraclones, compared with populations with a higher Dsg3 expression. Moreover, stringent sorting of populations showing both integrin b1-bright and Dsg3-dull expression enables even further enrichment of epidermal stem cells. These findings provide the basis for a new strategy for epidermal stem/progenitor cell enrichment, and encourage further study of the role of desmosomes in stem cell biology.

0492

Trichohyalin functions in specialized epithelial tissues as a keratin intermediate filament matrix protein and as a cell envelope barrier reinforcement protein

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Trichohyalin is expressed in specialized epithelia that are unusually mechanically strong, such as the inner root sheath cells of the hair follicle. We have previously shown that trichohyalin is sequentially subjected to post-synthetic modifications by peptidylarginine deaminases which covert many of its arginines to citrullines, and by transglutaminases which introduce intra- and inter-protein chain cross-links. Here we have characterized in detail the proteins to which it becomes cross-linked in vivo in the inner root sheath of the mouse hair follicle. We show that it serves two principal roles. First, it serves as an interfilamentous matrix protein by becoming cross-linked both to itself and to the head and tail end domains of the unique inner root sheath keratin intermediate filament chains. A new antibody reveals that arginines of the tail domains are modified to citrullines before cross-linking which clarifies previous studies concerning the filament chain composition. Second, trichohyalin serves as a reinforcement protein of the cornified cell envelope of the inner root sheath cells by becoming cross-linked to several known barrier function structural proteins, and in turn is cross-linked to the keratin filaments. Together, our new data document that trichohyalin is a multifunctional cross-bridging protein that functions in the inner root sheath and perhaps in other specialized epithelial tissues by conferring to and coordinating mechanical strength between their peripheral cell envelope barrier structures and their cytoplasmic keratin filament networks.

0493

Notch-1 mediated signaling increases IKKα levels--a critical determinant controlling keratinocyte terminal differentiation and cell survival

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Epidermal formation depends on a balance between cell proliferation, differentiation, and death. While deletion of IKKα gene blocks terminal differentiation in mice, biochemical pathways regulating IKKα in epidermis are unknown. Notch receptors regulate cell fate in response to ligands such as Jagged-1. In normal skin, Jagged-1 is expressed by keratinocytes (KCs) in basal cells and stratum spinosum, but not in granular cell layer or by corneocytes. To explore a role for Notch signaling in epidermopoiesis, human KCs were infected with retroviral constructs containing truncated (constitutively activated) forms of Notch-1 receptor (N^{IC}-1) and Notch-4 receptor (N^{IC}-4). Compared to empty linker control infection, which had no effect on KCs, infection with either N^{IC}-1 or N^{IC}-4, triggered growth arrest accompanied by stratification. Biochemical analysis confirmed induction of Notch-target genes such as Hes-1, and cell cycle regulator p21, as well as early/late differentiation markers (involucrin-loricrin). Importantly, only N^{IC}-1 triggered accumulation of IKKα accompanied by decrease in pro-apoptotic PKCδ, whereas neither N^{IC}-1 nor N^{IC}-4 induced IKKβ levels. By contrast, cytokines such as IFN-γ plus TNF-α induced IKKβ but not IKKα. When Notch signaling was interrupted by addition of a γ-secretase inhibitor (GSI), activated Notch receptor levels were reduced, and KCs underwent apoptosis in a concentration and time dependent fashion blocked by addition of caspase inhibitors. Apoptotic machinery involved in GSI-induced cell death included activation of caspase 3 and release of cytochrome C from mitochondria. Given the critical role for IKKα in terminal differentiation, we suggest that Notch-1 mediated signaling is responsible for endogenous IKKα accumulation. Furthermore, abrupt loss of Jagged-1 in upper epidermal layers may trigger cell death necessary for cornification following terminal differentiation. Thus, we propose Notch signaling regulates KC proliferation, differentiation, and cell death.

0494

Human epidermal glucosylceramides as major precursors of stratum corneum ceramides

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Not only as bulk constituents of ceramides (Cers) but also for their molecular heterogeneity which comprises of eight species is important for epidermal permeability barrier function in the stratum corneum (SC), we previously reported the structures of acylGlcCer and SM in epidermis. To further delineate the role of glucosylCer (GlcCer) as potential precursors for SC Cer, we here demonstrated the structures of human epidermal GlcCer species by gas chromatography-mass spectrometry, fast atom bombardment-mass spectrometry and nuclear magnetic resonance. Epidermal GlcCers comprise six distinct molecular groups, GlcCers 1-6. GlcCer 1 contains sphingenine and non-hydroxy (OH) fatty acids (FA), GlcCer 2, phytosphingosine and non-OH FAs, GlcCer 3, phytosphingosine with one double bond and non-OH FA, GlcCer 4, sphingenine and α-OH FA, GlcCer 5, phytosphingosine and α-OH FAs, GlcCer 6, phytosphingosine with one double bond and α-OH FA. Next, acylGlcCers and GlcCers were treated with β-glucocerebrosidase and the Cers released were compared with SC Cers. Taken together with our prior studies on epidermal SMs, all Cer species are derived from GlcCers and fractions of Cers 2 and 5 are from SMs.

0495

Pentagonal symmetry in proliferating epidermal keratinocyte colonies: a topological model for growth control

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Phase contrast microscope observations of serum-free cultures of normal human keratinocytes reveal a high frequency (>90%) of pentagonally-shaped colonies over a wide range of colony sizes that persist over many sequential cell generations. Macroscopic colonies derived from single cell isolates also display pentagonal colony morphology. Pentagonal symmetry in two-dimensions (2-D) was confirmed by a photographic technique known as "Markham rotation" where the order of reflective symmetry of an object is enhanced by super-imposition of additional images of the object by sequential rotation of the image around its center of gravity at a constant angular displacement. Best-fit for 5-fold symmetry was confirmed for colonies ranging from 4 to 59 cells. Strict adherence to pentagonal symmetry is lost upon coalescence of individual colonies in confluent monolayer cultures and instead, a "Chinese mosaic" of fours-fives-and sixs appears. Proliferating HaCat keratinocytes growing in serum medium also display a high frequency of 5-sided colonies. Finally, a review of published photographs of other epithelial cell type indicates the presence of pentagonal shaped colonies. The significance of these observations is not yet apparent, but one might speculate that proliferating cells possesses bilateral symmetry rotated around a perpendicular axis, dividing left and right sides, that runs from one vertex (head-end) and bisect the opposite base side (tail-end), thus, creating a five-sided figure. Evidence for this postulate comes from observations of retracting cell-cell contacts. Close packing of cells normally distributed in size according to their cell cycle proportions during rapid (GT=24hrs) asynchronous exponential growth is compatible with the construction of five-sided colonies. Finally, it is predicted that switching from growth to differentiation may involve a change from five-sided open path networks to 3-, 4-, and 6-sided closed path networks.

0496

The immunohistochemical analysis of the Myc/Mad1 expression in psoriasis

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Mad1 is a Myc antagonist that heterodimerizes with Max and functions as a transcriptional repressor. Recent observations indicated inhibition of proliferative activity subsequent to Mad1 overexpression has been demonstrated in a variety of cell types, including fibroblasts, keratinocytes, neural cells, haematopoietic cells. To elucidate whether c-Myc/Mad1 proto-oncogenes could be involved in the dysregulation of psoriatic keratinocyte, in situ expression of Myc/Mad1 protein was investigated immunohistochemically. Biopsy specimens were obtained from 12 patients with psoriasis vulgaris and from 7 healthy volunteers. Clinically stable psoriatic plaques of diameter 2-4 cm were biopsied from centre to the perilesional uninvolved skin. Specific anti-human c-Myc mouse monoclonal antibody (9E10) and rabbit polyclonal antibody against Mad1 (C-19) were used. Positive fluorescence signals were analysed using a confocal laser scanning microscope. In normal healthy skin, c-Myc was weakly positive in the nuclei of basal keratinocyte, whereas Mad1 was expressed in the cytoplasm of suprabasal layers. In fully-developed psoriatic epidermis, c-Myc expression was detected, but similar staining intensity to that of normal skin. In contrast to the normal and fully-developed psoriatic epidermis, c-Myc positive cells were increased in perilesional psoriatic epidermis. Mad1 expression was dramatically reduced in the fully-developed psoriatic lesions compared to perilesional uninvolved lesion. These findings suggested that Myc/Mad1 expression could be closely linked with the proliferation/differentiation disorder of psoriatic lesional keratinocytes. Although the role of c-Myc have not been completely elucidated in human epidermal cells, regulatory mechanisms of c-Myc/Mad1 could provide a clue for both pathological and therapeutic approaches of psoriasis.

0497

S100 genes: identification of a novel one and their expression in keratinocytes

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"Fused gene" subgroup of S100 family such as trichohyalin, repetin or fillagrin having S100 domain at the N-termini is expressed with differentiation in keratinocytes. However, expression of S100 proteins themselves have not been fully investigated in keratinocytes. So far, seventeen S100 genes have been identified in human. The fact that many human S100 genes are clustered at chromosome 1q21 (epidermal differentiation complex) implicates that they are involved in differentiation of keratinocytes. Thus, we investigated mRNA expression of seventeen human S100 genes (S100A1 to A14, S100B, S100P and S100Z) by means of RT-PCR in normal human keratinocyte cultured at various extracellular Ca++ concentrations. At extracellular Ca++ concentration of 0.05mM(basal condition), transcripts of S100A2, A4, A6, A8, A9, A10, A11 and A14 were detected. By elevation of extracellular Ca++ concentration to 2.00mM(72 hours:calcium induced stratification), expression of S100A13 and S100P mRNA was induced. Moreover, we found ESTs coding a novel S100 gene (mouse and human) in Unigene database (NCBI, U.S.A) as frequently expressed ones in libraries of skin. By RT-PCR, its transcript in normal human keratinocytes was equally and strongly detected at the extracellular Ca++ concentration of 0.05mM, 0.15mM and 2.00mM. Northern blot analyasis in tissues showed that the transcript was expressed in various tissues but most abundantly in the skin. These results suggested that some S100 proteins are involved in differentiation or stratification of keratinocytes.

0498

The head domain of keratin 5 binds to a dynein light chain, the cytoplasmic motor cargo complex, and might be involved in the distribution of keratin filaments and melanosomes

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Epidermolysis bullosa simplex with mottled pigmentation (EBS-MP), a rare variant of EBS, is distinctive with its hyper- and hypo-pigmented macules besides mild blister formation. All EBS-MP cases are caused by the missense mutation occurred at the 25th proline residue in the early head domain of keratin 5 (K5) so far. However, the etiological mechanisms, how this mutation located outside the rod domain should lead to the blisters and pigment anomaly in EBS-MP, are not yet understood. In this study, we tried to identify a protein, which binds to the head domain of K5 using yeast two-hybrid system. Fifty amino acids stretch of the critical K5 head region was used as bait to screen a human skin cDNA library. A tctex-1, a component of the cytoplasmic dynein light chains (DLC) was isolated. Dynein is a microtubule-based molecular cargo complex, and works for the directional transport of cytoplasmic proteins or organelles. DLC as well as dynein intermediate chain (DIC) were also co-immuno-precipitated with the antibody against K5 from cultured keratinocytes in vitro. We then prepared several truncated or modified bait constructs based on K5 head sequences, and found that the 25th proline was essential for the binding with DLC at least in the yeast system. In the cultured human keratinocytes, DLC and DIC were observed to spread granularly in the entire cytoplasm and partly aligned with keratin filaments especially in the recovering process from the replating. After co-cultivation of melanocytes and keratinocytes, melanosomes transferred in the keratinocytes were occasionally observed to co-localize with dynein complex along the keratin filaments. Our observation suggests that dynein could work for the distribution of K5 as well as melanosomes along microfilaments under particular condition. The disturbance of the binding of dynein and keratin filaments might cause the pigment anomaly and blisters noticed in EBS-MP.

0499

A reconstructed innervated skin-equivalent : a new tool for investigating the dermo-cosmetic product properties

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Somatic neurons transfer sensitive information from the skin to the central nervous system. In response, neuropeptides are delivered to epidermal cells. The release of neuropeptides such as CGRP from sensory neurons to skin cells leads to either cell proliferation and cell differentiation or an inflammatory response via the secretion of cytokines and growth factors. In order to reproduce this phenomenon, Bio Expertise technologies has developed an in vitro innervated skin model into a three compartmented culture chamber. A first compartment contains a reconstructed epidermis from human keratinocytes. A second compartment contains a culture of sensory neurons from rat dorsal root ganglio. Epidermis and sensory neurons are separated by a third compartment constitued of a collagen matrix retracted by human fibroblasts as a dermal structure equivalent. Histological analyses have shown a reconstructed epidermis with 8-9 layers of keratinocytes including a basal layer, a stratum spinosum and a stratum corneum. Laminin, transglutaminase, filaggrin, and cytokeratins are visualized by immuno-staining. Sensory neuron cell bodies are identified using an anti b tubulin antibody. Neuritic fibers which cross the biological dermal matrix and which establish a network among differentiated keratinocytes are stained with a neurofiber antibody. The neurons were mature and functional, as demonstrated by the spontaneous release of the CGRP neuropeptide. This new model offers an alternative method for testing the nerve-repairing capacity of molecules and the soothing or anti-inflammatory properties of products that are applied either locally or systemically.

0500

Skin interposition-grafting onto the chorioallantoic membrane of chick embryo as a model for wound healing studies

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There has been a struggle in recent years to develop a model that is closer to in vivo models, in order to replace the animal testing needed for studying the different mechanisms of the skin and the efficacy of ingredients. In this area, skin wound healing and repair studies are mainly performed on animals, due to poor in vitro substitutes to the complete structure of the skin and its interactions. Moreover, as our studies have shown that the use of an interposition technique to graft human skin onto the chorioallantoic membrane (CAM) of chick embryo is a reliable model for human skin studies, here we applied this technique for studies on human skin wound repair, and compared this method to an ex vivo model. Three human skin biopsies were grafted by interposing them between the shell and CAM membranes of 7-day old chick embryos. Parallel studies were performed using an ex vivo method. All samples were kept in the incubator for 3 or 6 days, and then removed and paraffin embedded for histological studies. Our results show that within 3 days, grafted skin samples showed greater repair activity than ex vivo samples, and that after 6 days, the repair was more complete and remarkably better in the grafted skin. Good dermal repair and scar formation was consistently found in grafted skin samples, with clear penetration of the dermal side by chick blood network that clearly improved the quality and preservation of the skin. In contrast, in ex vivo samples dermal scarring was partial, poor and only found in less than 40% of the samples. Epidermal cicatrisation was less obvious, but was found higher in grafted skin than in ex vivo samples. These results suggest that this method of interposition-grafting of multiple human skin biopsies onto the chick embryo, presents an interesting model for studying wounded skin repair, and pushes us a step further toward alternative methods to animal testing

0501

Recombinant type VII collagen sustains proliferation of the cultured keratinocytes

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Background: There is little information what happens on keratinocytes when they are exposed an excess amount of the recombinant Type VII collagen (rCOLVII). To compare the proliferation rate of normal human keratinocytes (NHK) and normal human fibroblast (NHF) after the addition of rCOLVII. The rCOLVII was produced in the COL7A1 cDNA transfected with HaCaT and evaluated for its ability to modulate the cultured cell proliferation. The rCOLVII expressing HaCaT cell line was generated by FLP/FRT system (The Flp-In system, Invitrogen Corp., Carlsbad, CA). The secreted rCOLVII into cultured medium was confirmed by western blot and was concentrated by microconcentrater at the rate of 1:30. The cultured medium of HaCaT transfected with control plasmid was similarly concentrated and regarded as a control additive. The NHK or NHF were seeded in 96 well ELISA plate and cultured for 24 hours in DMEM with 10% FCS supplemented with rCOLVII or control additive. The cell proliferation rate was non-radioactively assayed using Cell Titer 96 (Promega, Madison, WI). The absorbance at 570nm was recorded by 96 well plate reader (Model 450 Microplate Reader; Biorad, Hercules, CA), one hour after the addition of the assay reagent. The NHK supplemented with rCOLVII sustains proliferation comparing to the control. On the other hand, the NHF supplemented with rCOLVII did not show great differences in its proliferation. Addition of rCOLVII may have a positive influence on the proliferation of cultured keratinocyte. The proliferation of NHF was not much influenced by supplemented rCOLVII.

0502

Antisense oligonucleotides inhibit androgen receptor expression and biological activity in epithelial skin cells

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Androgen effects are mediated by their binding to nuclear androgen receptor (AR) molecules available in skin cells. The latter are able to synthesize steroid hormones *de novo* which are mostly activated in sebocytes and degraded in keratinocytes. Androgen activity *in vivo* can be inhibited by systemic administration of AR antagonists or inhibitors of androgen-metabolizing enzymes. In this study we describe a novel strategy, namely the inactivation of AR mRNA by antisense oligonucleotides. The human facial sebaceous gland cell line SZ95, which exhibits similar binding constants for AR with primary genital fibroblasts, and human preputial keratinocytes were used as substrates. Several antisense oligonucleotides were constructed and tested by applying various cell transfection systems *in vitro*, whereas AR protein expression was sufficiently inhibited by thioat oligonucleotides and 2'-O-methyl-RNA-antisense molecules using a cationic lipid-mediated transient transfection system. Effective transfection was visualized by intracellular detection of fluorescein-labeled antisense oligonucleotides. A concentration-dependent AR inhibition of up to 89% in SZ95 sebocytes and 25% in keratinocytes could be detected 17 hrs after 4h-transfection by western blot analysis. The effect was not due to inhibition of the motility of the AR-ligand complex, since translocation of AR from cytoplasm into the nucleus could be detected. The transient effect was confirmed by annulation of the inhibitory effect after 24 hr recovery time. Transfection had to be repeated to reestablish the effect. Biological androgen activity, namely an increase of epithelial cell proliferation and differentiation, was inhibited significantly after transfection with the antisense oligonucleotides against the AR.

0503

Many faces of periplakin: specific antibodies recognize periplakin in basal cell layer of epidermis

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Periplakin was first identified as a precursor of the epidermal cornified cell envelope, mainly residing in desmosomes and in interdesmosomal plasma membrane of differentiated keratinocytes, especially within the outer layers of epidermis. We originally isolated periplakin cDNA through yeast two-hybrid screening, using BPAG2/BP180/type XVII collagen intracellular domain as a bait. The physiological importance of this interaction has remained unclear, especially when related to the known expression pattern of periplakin in epidermis. However, the antibodies used so far have been raised against the C-terminal peptide of periplakin, the region, which is heavily involved in protein-protein interactions. Recently, the interaction of periplakin C-terminus with keratins, vimentin, periphilin, protein kinase B, and mannose-6-phospate receptor 300, has been reported. In order to avoid the false negative results in periplakin localization due to the epitope masking, different regions of periplakin were selected as antigens. Polyclonal rabbit antiserum against periplakin head domain (1), a mouse monoclonal antibody, recognizing the rod domain (2), and mouse polyclonal antiserum against the tail domain (3), each revealed a unique staining pattern in human epidermis. We conclude that periplakin head domain is accessible throughout the epidermis for the antibody recognition, while the rod domain is readily accessible only in the basal cell layer. The tail domain is masked in the basal cell layer by the protein-protein interactions, but is readily accessible in the outer layers of epidermis, where the rod domain is cross-linked to ceramides and is responsible for assembly of periplakin to the cornified cell envelope. We observed that while the soluble periplakin becomes lost after the detergent extraction, the rod domain becomes unmasked. Multiple localizations and protein-protein interactions indicate a role for periplakin as multifunctional cytolinker, similar to that established for plectin.

0504

Novel epidermal protein, periphilin

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While keratinocytes go through the terminal differentiation in stratified squamous epithelia, multiple proteins become cross-linked to form an insoluble layer of proteins, called the cornified cell envelope. To initiate formation of this envelope, periplakin and envoplakin, members of plakin family of proteins, bind to involucrin to form the scaffold on which several other proteins are assembled. Recently, we identified a novel epidermal protein, when the periplakin carboxyl-terminus (a.a 1548-1756) was used as a bait in a yeast two-hybrid screening. The protein was designated periphilin1. Periphilin locus in human chromosome 12q12 (PPHLN1) is composed of at least 11 exons which span about 110 kb. The open reading frame of 1122 base pairs encodes a polypeptide of 374 amino acids. The EST-database search demonstrated the possibility of alternative splicing at the 5′-end exons 2, 4, and 7, that can give rise to multiple protein isoforms. The PCR analysis of MTC-panels revealed the transcript of this novel protein, in addition of keratinocytes, in skeletal muscle, placenta, and heart. The carboxyl-terminus contains two helical regions resembling H2A and H2B regions in keratin proteins and shows homology to vitellogenins, a family of lipid binding proteins. Monoclonal antibody against the carboxy-terminal domain of periphilin stains the cell surface of keratinocytes in the outer layers of epidermis, showing co-localization with periplakin. Periphilin is also detected in nuclei throughout the epidermis. Various extraction procedures demonstrated the high insolubility of this protein, indicating possible cross-linking to a series of other proteins. Also, the sensitivity of periphilin to trypsin in keratinocyte cultures, together with the diffuse immunofluorescence staining at the surface of differentiated keratinocyte layers, suggest that periphilin may be secreted from the differentiated keratinocytes. In conclusion, the localization pattern and insolubility of this protein indicates that we have characterized a novel component of the epidermis.

0505

Human skin grafting onto chorioallantoic membrane of chick embryo, using the interposition technique: histological studies and comparison to an *ex vivo* model

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Different in vitro skin substitutes have been developed in recent years for the purpose of decreasing animal testing and moving closer toward in vivo evaluation of the toxicity of products and efficacy on the skin. Nevertheless, these methods are expensive and often incomplete. Moreover, ex vivo method is limited to short-term studies . A few recent studies have shown that skin samples, or cancer xenografts survived grafting onto the chorioallantoic membrane (CAM) of chicken embryo. Therefore, we were interested in developing this technique further in order to enable longer and better survival of skin biopsies. In order to do this, we used an interposition technique where 3 mm punch biopsies of human skin were interposed gently in between the shell and CAM membranes of 7-day old embryos. The eggs were kept in an incubator for 6 days, after which the membranes with the grafted skin samples were incised and submitted to histological studies. For comparison and controls, parallel studies included placing skin biopsies on the CAM after removal of the shell membrane, as well as the culture of skin samples following ex vivo method. These studies showed that skin grafted using the technique of interposition is markedly better than skin grafted by placement on the CAM, especially concerning the epidermis and keratin layer. The grafted skin developed a nice interaction with the CAM, which provided new evidence of aided skin nourishment. The grafted skin showed a remarkable improvement and preservation over the ex vivo skin, with great structural preservation even after 6 days. Our results confirm that skin grafting onto chick embryo is a very interesting method, and the results show that the new interposition technique clearly improves grafted skin quality. This technique offers an interesting test method for skin investigations in the field of advanced in vitro methods and alternative methods to animal testing.

0506

Fourier transform infrared microspectroscopy imaging of endogenous and exogenous molecules in skin sections

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Fourier transform infrared (FTIR) imaging microspectroscopy is a relatively new technology that provides the opportunity to directly map the distribution of molecular components within a biological sample, while maintaining sample architecture. In this approach the full information content inherent in an infrared spectrum of a biological sample (such as a skin section) is directly coupled with spatial information. In the currect experimental design a 400 x 400 micron sample area is directly mapped to an array detector such that 4096 IR spectra are simultaneously generated with each spectrum corresponding directly to a specific 7 x 7 micron sample section. In recent studies this technology has demonstrated great promise for mapping molecular architecture and chemical composition in pathological and normal bone sections. This presentation will briefly describe FTIR spectroscopic imaging technology and present recent data from our experiments with various skin sections, including studies of transdermal delivery. In the transdermal delivery experiments we have imaged the distribution of penetrants in skin and compared these with images of endogenous molecules, thereby providing direct spectroscopic images of penetrant location within skin. The current experimental approach permits data to be acquired in a matter of minutes and without the use labelled molecular probes (such as fluorophores). This presentation will highlight our initial studies, which clearly demonstrate the potential of imaging FTIR microspectroscopy to directly map the distribution of exogeneous molecules in skin sections, as well as providing chemical composition images of endogeneous molecules across a skin section.

0507

Differentiation-dependent localization of exocytosis machinery in epidermis suggests a role in lamellar granule trafficking

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Mechanisms for transport of lamellar granule (LG) lipids to the site of barrier formation at the junction of the uppermost layer of the viable epidermis and stratum corneum are not understood. Electron microscopic evidence suggests that LGs originating from Golgi membranes fuse with granular cell plasma membranes and extrude their contents into the extracellular compartment by exocytosis. This scheme has recently been challenged by the cubic model of lipid trafficking in which the machinery and energy to drive membrane fusion and exocytosis are not invoked. To elucidate molecular processes involved in LG trafficking we examined human epidermis by confocal immunofluorescence microscopy for the presence and location of proteins believed to mediate exocytosis in neuronal and other specialized secretory cells. Antigens expressed in epidermis include: several annexins (calcium and phospholipid binding proteins that promote membrane fusion); synaptotagmin (involved in calcium-dependent exocytosis); NSF, VAMP-1/2, Vtip1, SNAPs, and several syntaxins (components of the SNARE complex); SCAMP-1 (mediates vesicle/plasma membrane fusion in association with SNAREs); synapsin I (pre-fusion vesicle tethering protein); and Sec 3, 6, 8, and the guanine nucleotide binding protein Ral A (components of the exocyst complex, required for polarized exocytosis). Annexins 2 and 7, syntaxin 3, SCAMP 1, synapsin 1, and Ral A are preferentially distributed at the junction of the stratum granulosum and stratum corneum and are partially colocalized with the LG markers AE17 antigen and glucosylceramides. These results show that proteins important in vesicle/plasma membrane targeting and exocytosis are expressed in epidermis. The differentiation-dependent distribution of several of these antigens and partial colocalization with known LG components suggests their participation in a major secretory pathway of terminally differentiated keratinocytes, the delivery of LG cargo for epidermal barrier assembly.

0508

Carnitine is synthesized in the epidermis

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Carnitine is an essential cofactor in the transport of long-chain fatty acids across the inner membrane of mitochondria, and thus plays an important role in energy metabolism via β-oxidation. In mammals, carnitine homeostasis is maintained by endogenous synthesis and exogenous intake from dietary sources. Carnitine synthesis has been thought to take place exclusively in the liver, kidney and brain. Other tissues depend on active uptake via carnitine transporter. The aim of this study was to clarify the homeostasis of carnitine in the epidermis, a tissue wherein the transport and biosynthesis of carnitine remain obscure. Our findings as follows suggested that carnitine also synthesized in the epidermis. Carnitine transport was defective in mice with juvenile visceral steatosis (JVS), though the actual levels of carnitine and epidermal function in the JVS mice were not different from those wild-type littermates. In cultured normal human keratinocytes, reverse transcription polymerase chain reaction (RT-PCR) confirmed the gene expression of enzymes for carnitine biosynthesis; three enzymes for carnitine biosynthesis were expressed (trimethyllysine hydroxylase, trimethylaminobutyraldehyde dehydrogenase, butyrobetaine dioxygenase). These observations confirmed that carnitine is synthesized in the epidermis.

0509

Effects of a moisturizer containing dimethicone and high levels of glycerin on keratinocyte proliferation and epidermal architecture

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Improved barrier function and skin appearance are well described effects of skin moisturizers. Dimethicone, an ingredient now found in many skin care products, has been shown to protect against irritant injury and improve hand dermatitis. The purpose of this study was to assess the effects of twice daily application of a moisturizer containing dimethicone and high levels of glycerin on keratin expression, epidermal architecture, and barrier function in normal-appearing skin. A 4mm punch biopsy was taken at baseline and after 28 days of bid application of a moisturizer containing dimethicone and high levels of glycerin from photodamaged forearms of 25 women. Post-treatment biopsies were randomly assigned to be taken from the same arm or the opposite arm. Twenty-four subjects completed the study. At baseline, keratins 10 and 14 were expressed in all specimens, and k6 and k16 were not expressed in any specimen. At day 28, k10 and k14 were again expressed in all specimens. Keratin 6 was expressed in 14 (58%) specimens and k16 in 19 (79%) specimens. No difference between the pairs where the biopsies were taken from the same or from the opposite arm was evident. Maximal epidermal thickness, adjusted for baseline value and side, increased 0.017mm (28%) from baseline (P<.001). There was no change in the appearance of the stratum corneum or granular layer. Trans epidermal water loss decreased 0.55 (13%) from baseline, (p=0.03). Induction of keratins 6 and 16, commonly associated with keratinocyte proliferation and wound healing, was associated with increased epidermal thickness and improved barrier function in skin after 28 days of treatment. This finding suggests that even clinically normal appearing skin may benefit structurally from routine use of moisturizers containing dimethicone and high glycerin levels.

0510

Permeablity of sodium ascorbic acid-2-O-phosphate into the human skin and its scavenging of intradermal free radicals

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Sodium ascorbic acid-2-O-phosphate (APS) has been widely utilized as a skin brightening agent in cosmetics. Recently, we reported APS exerted a remarkable efficacy for acne. In this study we investigated its skin-permeability and scavenging of free radicals to reveal the mechanism underlying this efficacy. When applied onto the skin, APS permeated into the epidermis and was quickly converted to ascorbic acid (AsA). Each tiny human skin piece devided out of one biopsy from a volunteer was placed in a modified Bronaugh's diffusion chamber, with 2.0% APS, AsA, or ascorbic acid-2-O-glucoside (AG) applied on it, and incubated for six hours. Then the isolated epidermis was homogenized and the contents of AsA, APS, and AG were determined by HPLC. The epidermal AsA content was the highest (2.8 nmol/mg protein) in APS treated skin, while it remained 0.4 and 0.6 nmol/mg protein in AsA- and AG-treated skin, respectively. The unconverted derivative contents were similar (1.0 to 1.2 nmol/mg protein) in AG and APS treated skin, which indicated that the hydrolysis rate of APS was much higher than that of AG. APS showed an excellent capability of quenching free hydroxyl radicals in the skin. A human skin cell culture model (TESTSKIN) was pretreated with 200 mM AsA, APS or AG for two hours, and the intradermal ESR spectrum was measured by DMPO spin-trapping method under UVB irradiation. The amount of generated hydroxyl radicals was significantly reduced in APS-treated culture, to 25 percent of non-treated control. AG showed almost no scavenging due to its slower permeation and conversion to AsA. We believe those observations well explain the improving effects of APS on acne in clinical studies, in consideration along with other biochemical activities of APS including enhancement of collagen synthesis and depigmentation.

0511

A novel type I keratin-binding protein, okuribin, acts as a linker between keratin filaments and the nuclear envelope

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Keratin intermediate filaments are the major cytoskeletal proteins in the epithelial tissues, which are widely distributed all through the cytoplasm. At the cell periphery keratins are bound to the desmosomal components such as desmoplakin, however, proteins, which would link the keratin filaments to the cytoplasmic organelles, are not yet identified. We thus tried to characterize proteins that interact with keratins by yeast two-hybrid system. Employing the head and tail domains of epidermal type I and type II keratins as baits, we made several rounds of screening of the human skin cDNA. A novel protein termed okuribin was cloned as a type I keratin binding protein. Okuribin directly binds with the head domain of type I keratins K14, K16 and K18 but not with type II keratins. The okuribin mRNA in size of 4.1 kilo-nucleotides was expressed in the various epithelial tissues including skin as well as non-epithelial tissues like muscles. The okuribin protein predicted as a105 kDa protein encoded by 950 amino acids possessing eight nuclear localization signals as well as a signal peptides and one putative transmembrane segment. When the okuribin cDNA was fused with green fluorescence protein (GFP) or flag tag at its carboxyl terminus, and used for the forced expression into cultured keratinocytes, GFP and flag signals were observed predominantly along with the nuclear membrane. We then prepared an antiserum against the synthetic 20 peptides corresponding to the central region of okuribin. The endogenous okuribin in keratinocytes was observed mainly at the outer nuclear envelope, and occasionally in the nuclei depending on the maturation of the nuclear envelope verified by the staining intensity of lamin. The matured nuclei, where lamin and okuribin were observed strongly at the inner and outer envelop, tend to be tightly surrounded or enclosed by the keratin network in the immune-electron microscopy. From these data, okuribin is considered to act as a missing link connecting the keratin filaments and juxta-nuclear space.

0512

Suppression of hyaluronan synthesis by 4-methylumbelliferone retards keratinocyte migration and proliferation

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Hyaluronan is a glycosaminoglycan abundant in the extracellular matrix of basal and spinous layers of the epidermis, participating in normal keratinocyte proliferation and differentiation. In general, high levels of tissue hyaluronan are associated with enhanced migration of cells, for example in healing wounds, but also in aggressively growing tumours. Recent studies have used 4-methylumbelliferone (MU) as a hyaluronan synthesis inhibiting agent in fibroblasts, but little information about the mechanism of this inhibitory effect is available. We used a newborn rat epidermal keratinocyte cell line (REK) to investigate the effect of MU on the hyaluronan metabolism and its biological consequencies. We found that MU (0.2-1 mM) has a dose dependent inhibitory effect on hyaluronan secretion of these cells. At the same time the REK cells lost their typical polarized morphology, their proliferation and migration was slowered and adhesion to substratum increased. The RT-PCR showed that the diminished HA secretion level was related to reduced hyaluronan synthase (Has2 and Has3) mRNA expression levels. This reduction was observed after 1 hour treatment, excluding the possibility of a secondary effect. MU had no effect on the mRNA expression of the hyaluronan receptor CD44. The results of this study support the idea of MU as a useful agent for relatively specific inhibition of hyaluronan synthesis and establish the role of Has2 and Has3 in this inhibition. MU has potential for clinical conditions that would profit from inhibition of excessive hyaluronan production.

0513

Induction of apoptosis in keratinocytes by mutant transglutaminase 1 lacking a Cys-cluster for membrane anchoring

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Transglultaminase 1 (TGase 1) is essential to formation of cornified envelope and development of skin barrier. The function of TGase 1 is possibly achieved by membrane-anchoring of the enzyme via fatty acid thioesterification of a Cys-cluster near the N-terminus. Indeed, a mutation in the Cys-cluster has been reported in a case of autosomal recessive congenital ichthyosis, although the effect of the mutation has not been delineated. In this study, to elucidate the biological role of the Cys-cluster in keratinocytes, we visualized the intracellular location of TGase 1 by adenovirus-mediated expression of a TGase 1-GFP fusion protein. When cultured human keratinocytes were infected with recombinant adenovirus, a wild-type TGase 1-GFP was initially located around the nucleus and then distributed over the cytoplasm as granules and on the cell periphery. In contrast, a mutant TGase 1-GFP, the Cys-cluster of which is replaced with Ala, was distributed diffusely in the cytoplasm as well as around the nucleus and induced apoptosis of keratinocytes. Adenovirus-mediated expression of only GFP did not cause such apoptotic changes. These results suggest that the Cys-cluster of TGase 1 is involved not only in membrane anchoring, but also in sequestration of the enzyme. The restricted intracellular localization of TGase 1 may prevent keatinocytes from premature apoptosis due to unusual dislocation and activation of the enzyme during terminal differentiation.

0514

A keratinocyte outgrowth system useful as a new tool for analyzing proliferation, migration, and differentiation

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Normal human epidermal keratinocytes (KC) stop proliferation and differentiate at confluency. This contact inhibition is thought to be mediated in part by mechanical tension. To prove this, we created a new assay system. First, a 10mm diameter cloning ring was put on the center of the 60mm dish coated with type I collagen. Then KC were plated in the ring and incubated 4-16h. The cloning ring was removed, and the floating cells were washed away. Thus, we had a circular epidermal monolayer sheet only in the center of the dish. If we used SFM, whose Ca concentration is below 0.1mM, as a medium, the KC in the perimeter migrated individually, and the KC in the center portion started keratinizing. However, when we added calcium chloride to the SFM (final concentration more than 0.5mM), KC at the periphery showed marked lamellipodia without losing contact with the surrounding cells. These KC showed coordinate sheet-like outgrowth as a whole. After a few days, the diameter of the sheet increased 4mm/day constantly for several days without notable keratinization in the center. BrdU labeling experiments revealed that the distribution of BrdU positive cells gradually shifted from the center to the peripheral area. To maintain the expanding sheet structure, KC have to migrate and proliferate as if they are under precise control. Otherwise, there should be some free spaces or holes inside the sheet. The addition of cytocharasin D, an actin polymerizing inhibitor, abolished complete KC outgrowth, and, after washing out the inhibitor, the KC resumed outgrowth. These results suggest that actin polymerization is a driving force of this outgrowth. Addition of AG1478, a tyrosine kinase inhibitor, partially inhibited outgrowth, indicating that growth factor mediated signaling is also important for this outgrowth. Our culture system will help in understanding the mechanisms of cell migration, proliferation and differentiation. Using this assay system, we can learn more about wound healing, because this in vitro sheet-like outgrowth simulates an in vivo injured site.

0515

Calcium entry into keratinocytes induces cell surface expression of lysosomal membrane proteins

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During the final steps of epidermal differentiation, just before keratinocytes gain the stratum corneum as keratinized dead cells, extracellular calcium ions enter into these epidermal cells and induce transglutaminase activity and cornified envelope formation. An entry of calcium mediated by ionophores has been reported to induce exocytosis of lysosomes in other cell types. In this study, we investigated whether a similar phenomenon might occur during the final steps of keratinocyte differentiation. Using autocrine culture conditions, ionomycin treatment induces cornified envelope formation in keratinocytes, but also morphological changes including plasma membrane blebbing. No immediate alteration in cell viability can be detected using the ethidium bromide/acridine orange staining assay, however the measurements of cytosolic enzyme activities (dipeptidylpeptidase III and lactate deshydrogenase) in the culture medium suggest some membrane leakage, possibly associated with cell blebbing. Simultaneously, the activity of a lysosomal enzyme (cathepsin C) in the culture medium was increased upon ionomycin treatment. Using metabolic labeling followed by immunoprecipitation, we found that ionomycin induces release of processed as well as unprocessed forms of cathepsin D in the culture medium. These results suggest that lysosomal and prelysosomal components are also released upon ionomycin treatment. In order to assess whether this presence of lysosomal components in culture medium might result from organelle exocytosis instead of membrane leakage only, the cell surface expression of lysosome-associated membrane proteins (Lamps) 1 and 2 was compared between untreated and ionomycin-treated keratinocytes, using biochemical and morphological approaches. The appearance of both Lamps at the surface of ionomycin-treated keratinocytes suggests that calcium entry could stimulate significant exocytosis of lysosomes, simultaneously to terminal differentiation and possibly cell death.

0516

Regulatory mechanisms of eosinophil chemoattractant production by normal human keratinocytes

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Eosinophils play a crucial role in the inflammatory process in allergic diseases. Selective eosinophil chemoattractants (ECAs), such as eotaxins, are believed to be the key molecules produced at the inflamed sites that induce eosinophil accumulation. In addition, ecalectin (galectin-9) isolated from a human T-cell-derived expression library is a novel and eosinophil specific chemoattractant produced by several cell types, and could be also involved in this process. Keratinocytes are one of the major regulators of the skin immune system and represent targets of eosinophil attacks in allergic skin diseases. Keratinocytes may regulate eosinophil accumulation via the production of ECAs. To understand regulatory mechanisms of eosinophil infiltration into the epidermis, we investigated the expression of eotaxins (eotaxin-1, eotaxin-2 and eotaxin-3) and ecalectin in normal human keratinocytes. Normal human keratinocytes were cultured in serum-free medium with several cytokines, including 10ng/ml hrTNF-alpha, 10ng/ml hrIL-4, 10U/ml hrINF-gamma for 24hrs. RT-PCR analysis revealed that eotaxin-1 mRNA expression was up-regulated by the combination of TNF-alpha and IL-4 or IFN-gamma. Eotaxin-2 mRNA was constitutively expressed in these cells and no regulatory effect was seen with any types of cytokines tested. The expression of eotaxin-3 mRNA was induced by IL-4, but not enhanced by the combination of TNF-alpha or IFN-gamma. Finally, ecalectin mRNA expression was clearly increased when stimulated with INF-gamma. These results lead us to conclude that selective eosinophil accumulation in the epidermis can be induced by not only Th2-type cytokine stimulation through the production of eotaxin-1 or -3, but also Th1-type cytokine stimulation through eotaxin-1 or ecalectin. Moreover, eotaxin-2 may in part contribute to the eosinophil infiltration from the dermis to epidermis, as the production of eotaxin-2 by dermal fibroblast was below the detectable level.

0517

Inhibition of p53-activity in the skin is associated with an impaired terminal differentiation of keratinocytes and down-regulates Caspase-14 expression

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The tumor suppressor gene p53 plays a key role in the regulation of cellular responses to DNA damage. However, the involvement of p53 in the differentiation of the skin is still poorly understood. In this study we have investigated the effect of a chemical inhibitor of p53, α-Pifithrin (α-Pif), on keratinocyte differentiation and Caspase-14 expression in skin-equivalents (SE). After incubation of SE with α-Pif for 7 days we found a strong downregulation of p53 mRNA and protein, as well as the p53 downstream element, p21WAF1/Cip1. H&E staining of these SE revealed a parakeratotic phenotype. In addition electron microscopical analysis showed only few and small keratohyalin granules and few and underdeveloped odland bodies in α-Pif treated SE. Furthermore the basement membrane was virtually not present and the hemi-desmosomes are only partially present and the intercellular plaques are absent. When analyzing expression of Caspase-14 in SE, we found a strong reduction of Caspase-14 protein and mRNA by α-Pif. To study the effect of p53 inactivation on Caspase-14 expression in vivo, we studied skin biopsies of Bowens disease, actinic keratosis and condyloma accuminata, in which p53 is inactivated by mutations and HPV respectively, for Caspase-14 expression. In lesional skin of all three diseases Caspase-14 expression was strongly reduced, whereas it was not affected in adjacent skin. In conclusion, our results suggest that p53 plays an important role in the terminal differentiation of epidermal keratinocytes, and that it is directly involved in the regulation of Caspase-14 during this process.

0518

Retinoic acid up-regulates hyaluronan production but retards keratinocyte differentiation

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Vitamin A is known to influence epidermal differentiation and cause hyperproliferation. Our previous work has shown that growth factors stimulating keratinocyte proliferation and impairing differentiation induce epidermal hyaluronan production. Hyaluronan is the main extracellular matrix molecule in the vital cell layers of epidermis but it is virtually absent in the terminally differentiated cell layers. In the present work we studied the influences of vitamin A on epidermal morphology and hyaluronan content in organotypic keratinocyte cultures and mouse skin in vivo. We found that topical treatment of mouse epidermis with retinoic acid lead to a rapid increase in the epidermal hyaluronan content, concomitantly with increased expression of the hyaluronan receptor CD44, and hypertrophy of the epidermis. After 4 days treatment the ear epidermis was ~1.5 times thicker than the control epidermis, with hyaluronan and CD44 localizing in the basal and spinous cell layers. Organotypic keratinocyte cultures treated with retinoic acid became thicker than control cultures, and showed accumulation of hyaluronan in the basal and spinous layers, as well as increased CD44 expression. Differentiation was deficient in retinoic acid treated cultures, stratum corneum was thinner and the expression of K10 was diminished compared to control cultures. The data show that retinoic acid regulates hyaluronan metabolism in epidermis, and that the effect is targeted directly to keratinocytes since it can be reproduced in a culture model lacking other cell types. The data also confirm the previous findings that increased hyaluronan content correlates with delayed differentiation in epidermis.

0519

Sphinganine N-acyltransferase (SNAT) activity in the *de novo* pathway during proliferation and differentiation of human keratinocytes

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Ceramides (CERs) are a major component of the stratum corneum, accounting for 30-40% of stratum couneum lipids by weight. CER generation occurs either via reverse ceramidase or via sphingomyelinase (SMase), the de novo pathway of sphingolipid biosynthesis. The present study examines the level of sphinganine N-acyltransferase (SNAT) activity in the de novo pathway during the proliferation and differentiation of human keratinocytes. The materials used included biologically negligible amounts of N-heptadecanoyl CoA, to serve as the substrate, and a high concentration of defatted bovine albumin sphingosine complexes for use in the Vmax and HPLC thermospray-MS assays. SNAT activity (pmol/min/mg protein or μg DNA) was highest 24 hours after subculture. SNAT activity 48,72 and 96 hours after subculture tapered off gradually, compared with the drastic decreases 24 hours post subculture. Reverse ceramidase activity was very low compared with SNAT activity. A different time frame was required for the generation of each type of CERs. These results suggest that SNAT, like serine-palmitoyl transferase, plays an important role in generating CERs during keratinocyte differentiation.

0520

The dialog between keratinocytes and sensory neurons in the skin : approach using a simplified *in vitro* model

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There are numerous cell contacts between neuritic fibers and cutaneous cells, which has led to the concept of a neuro-cutaneous system. The cell bodies of sensory neurons are localised in the dorsal root ganglia on each side of the spinal chord. Somatic sensory neurons transfer sensitive information from the skin to the central nervous system. In response to inflammatory injury, there is an anterograde release of neuropeptides through nerve fibers toward the cell layers of the epidermis. In vivo, local release of neuromediators such as Calcitonin Gene-Related Peptide (CGRP) and substance P lead to modulate inflammatory reactions by keratinocytes. Neuropeptides specifically modulate the proliferation of inflammatory cells and the production of cytokines. In vitro, sensory neurons are mature after 9 days and release CGRP and substance P. Cultured with neuropeptides, human keratinocytes are stimulated and release inflammatory cytokines such as IL-8 and TNF-a. When neurons are cocultured with keratinocytes in 96 or 24 well-culture microplates it has been shown, using an enzyme immunoassay, that, the release of neuropeptides into the culture supernatant increases. In addition, the release of CGRP can be down- or up-regulated by incubating these cultures with specific compounds. Immunofluorescence staining performed directly in the wells with either anti-neurofilament (PGP 9.5) or anti-neuropeptide (CGRP) antibodies permit both to control the integrity of the neurons before dosages and to quantify staining modulation by image analysis. From the results obtained with several products, the use of this model within its limitations is proposed for the screening of molecules with either anti-inflammatory or soothing properties .

0521

Epidermal hyaluronan metabolism in CD44-negative mice

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Hyaluronan is a major matrix constituent between the proliferating basal cells and the differentiating spinous cells of human skin, and CD44 has been suggested to be its major cell surface receptor. We therefore studied the expression of hyaluronan in the epidermis of CD44 knockout mouse. In the mouse, epidermal hyaluronan concentration was developmentally regulated, being high during the fetal period and shortly after birth, but low in adult skin. This was in contrast to humans, where high epidermal hyaluronan expression remains throughout the life. The amount and localization of epidermal hyaluronan in the CD44 negative mice corresponded that of the wild type in embryonic and adult mice under normal conditions. In the wild type mouse, a mild trauma caused by tape stripping triggered a strong increase in the content of epidermal hyaluronan, concomitantly with epidermal hyperplasia and induction of CD44 expression. In the epidermis of CD44 -/- mice, the hyaluronan response to tape stripping was weaker than in the wild type mice. Despite the lower total hyaluronan content in CD44 negative epidermis hyaluronan was found in the upper spinous and granular cell layers which were hyaluronan negative in the wild type mice. Keratinocytes isolated from wild type and CD44 negative mice synthesized hyaluronan at about the same rate. However, the wild type cells retained more hyaluronen in the cell layer than the CD44 -/- cells. Histochemical staining confirmed this finding and showed that hyaluronan in the wild type cells was localized on the dorsal cell surfaces, while in CD44 negative keratinocytes hyaluronan was mainly located between the cells and the substratum. Our data show that adult mouse epidermis is characterized by a low but inducible hyaluronan expression. The expression of CD44 is induced concomitantly with hyaluronan upon wounding, and is necessary the proper organization of hyaluronan in the keratinocyte pericellular matrix.

0522

Delineating the role of PKCδ in skin proliferation, differentiation and wound healing utilizing PKCδ null mice

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PKCδ, a novel PKC isoform, is implicated in migration, proliferation and differentiation of keratinocytes. We have further delineated the role of PKCδ isoform in skin, by studying the characteristics of skin *in vivo* and *in vitro* utilizing PKCδ knockout (KOδ) mice. The KOδ mice are born viable and upon adulthood, males are infertile. However, skin gross appearance and morphology is normal. Mouse keratinocytes isolated from wild type (WT) and KOδ mice were characterized *in vitro*. In comparison to WT keratinocytes, KOδ cells attached poorly to the culture dish with plating efficiency reduced by 75%. WT keratinocytes fully spread 2-4 hours following attachment. In contrast, KOδ keratinocytes maintained a rounded morphology up to 24 hours following plating and cell migration was radically reduced. In addition, proliferation of KOδ keratinocytes as measured by thymidine incorporation was reduced by 70%, as compared to WT cells. This was associated with an increased differentiation potential of KOδ keratinocytes. Prior to Ca^{+2} elevation, KOδ keratinocytes but not WT cells expressed high levels of spinous markers keratin 1 and 10 with no change observed in expression pattern of Filaggrin, a granular differentiation marker. Expression of these markers was further increased following Ca^{+2} elevation. We have next subjected KOδ mice to wound healing studies. Full skin incisions were made on the back of mice and wounds were followed for 7 days. Impaired wound healing was observed only in KOδ mice but not in KOα or KOζ mice as analyzed by wound strength and histology of wound closure. Interestingly, KOδ skin expressed higher levels of spinous (K1) and granular (Loricrin) differentiation markers. Altogether, these results suggest PKCδ as a crucial mediator of keratinocytes attachment, spreading, proliferation and differentiation processes in skin *in vivo* and *in vitro*.

0523

Characterization of newly-exposed human wound keratinocytes

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Upon injury to the skin, keratinocytes exposed to various components from blood, cell products and extracellular matrix undergo dramatic changes in order to migrate across the wound bed and eventually close the wound. Historically, there have been two models proposed to explain epidermal wound closure: 1) the "sliding model" in which the basal keratinocytes actively migrate across the wound matrix and 2) the "rolling model" in which suprabasal keratinocytes actively roll over basal keratinocytes and tumble onto the wound matrix. We used immunohistochemical methods to label incisional human wounds of 6 hour, 1 day and 3 day duration to evaluate the keratinocytes along the wound margin that are in direct contact with the wound matrix. Antibodies included keratin markers K10, K14, K15, K16, cell surface integrin receptors alpha 2, alpha 3, alpha 5, beta 4, the uncleaved (precursor) form and the cleaved form of laminin 5. At the wound edge, a sub-population of suprabasal keratinocytes, defined by anatomical location, show differential expression of keratins K14, K15, K16, the cell surface integrin receptors alpha 2, alpha 3, alpha 5, beta 4, as well as the uncleaved and cleaved forms of laminin 5. With the exceptions of K10 and K16, all of these proteins are typically associated with basal keratinocytes. Our results suggest that these migratory suprabasal keratinoctyes at the wound margin are capable of rapid adaptation to the wound environment. We conclude that both suprabasal and basal keratinocytes are actively involved in epidermal wound closure allowing the largest possible pool of epithelial cells to participate in this process.

0524

Uptake and elimination of drugs and endogenous substances in cells of the human skin is an active process

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Normal human epidermal keratinocytes (NHEK) have been shown to express a cell type specific pattern of cytochrome P450 enzymes and efflux transport proteins showing that these cells metabolize and excrete a variety of xenobiotics. Recently transport proteins (organic anion transporting polypeptides, OATPs) involved in the uptake of xenobiotics have been detected and here we analysed the mRNA- and protein-expression profiles and functional activities of these proteins in NHEKs in comparison to primary liver cells. Constitutive expression of OATP -B, -D and -E was shown for the first time in NHEKs on a molecular level using RT-PCR and northern blot analysis, as well as in human skin tissue shown by tissue blot hybridization and immunohistochemistry. Expression of OATP-A and -C was not detected in any of the keratinocyte samples. In contrast, liver tissue showed a significant expression of OATP-A and -B as well as OATP-C, a weak expression of OATP-D and no expression of OATP-E. These data revealed that NHEK express a specific profile of transporters involved in drug influx. Using a newly developed uptake-transport assay, uptake and efflux of endogenous substances and xenobiotics like estradiol-17b-glucuronide and estrone sulfate was inhibited in NHEKs by specific inhibitors such as taurocholate or indomethacin, verifying the functional capacity of the expressed OATPs and MRPs. Human dermal fibroblasts seem to have a lower influx transport activity for estradiol-17b-glucuronide which correlates with the immunohistological data. Even though the substrate specificity of the OATP isoforms and MRPs is only partially known till now, our findings support the concept that uptake and elimination of large organic cations like drugs in keratinocytes is an active and cell type specific transport process.

0525

Function of the N-terminal calcium-binding domain in human profilaggrin

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Profilaggrin, a protein expressed in epidermal granular cells, consists of multiple filaggrin units and N- and C-terminal domains that differ from filaggrin. Profilaggrin is initially stored in keratohyalin granules and, as cornification proceeds, it is proteolytically processed by calcium-regulated proteases to generate the keratin-binding peptide filaggrin and a N-terminal peptide that contains a S100 calcium-binding domain. The N-terminal peptide (AB domain), once cleaved from profilaggrin, localizes to nuclei in epidermal granular and transition cells and shows a similar nuclear distribution when expressed in vitro (JID 119:661-69, 2002). To further understand the function of the N-terminal calcium-binding domain in regulating profilaggrin expression or processing, we mutated the key acidic residues in each S100 EF-hand and examined expression and processing of a profilaggrin minigene in vitro. Both control and mutant profilaggrin minigene constructs (consisting of FLAG-AB-5 filaggrin units) were expressed in COS-7 cells and rat keratinocytes. Expression was examined by both indirect IF and Western blotting. Abrogation of calcium binding did not affect either expression of the profilaggrin miniprotein, or formation of the keratohyalin-like granules observed in transfected cells in vitro. Studies are in progress to determine if calcium binding affects profilaggrin processing in cultured keratinocytes or in transgenic mice expressing this transgene.

0526

Comparative proteomic profiling of murine skin

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Mammalian skin is regularly exposed to different environmental stresses, each of which results in specific compensatory changes in protein expression that can be assessed by proteomic analysis. We have established a reference proteome map of murine skin allowing the resolution of greater than five hundred protein spots in a single two-dimensional polyacrylamide gel. Forty-four protein spots, corresponding to 28 different cutaneous proteins, were identified using MALDI-TOF MS and the Mascot online database searching algorithm. Twenty-five proteins were expressed at higher levels in the epidermis, whereas only 9 were found predominantly in the sub-epidermal tissues. Proteins of diverse function were identified, including those involved in apoptosis, growth inhibition, the maintenance of structural integrity, translational control, energy metabolism, calcium binding, cholesterol transport, and the scavenging of free-radicals. Five molecular chaperones including ER60, GRP78, HSP60, HSP70, and HSP27 were also identified. Of these, HSP27 expression was confined mainly to the epidermis, and expression of ER60 was found primarily in the sub-epidermal tissues. Proteomic analysis of skin following heat or cold shock resulted in increased levels of HSP27, HSP60, and HSP70 suggesting involvement of these chaperones in the cutaneous response mechanism to temperature stress. These data establish numerous reference markers within the proteome map of murine skin and provide an important framework for future efforts aimed at characterizing the epidermal and sub-epidermal responses to environmental changes.

0527

Epidermal stem cell frequency: a novel *in vivo* functional assay

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The field of hematopoietic stem cells has been greatly advanced by a quantitative, in vivo assay that relies on the long-term repopulating ability of stem cells to define them. Using the same concepts but different experimental parameters we developed an analagous repopulating assay to study stem cell function in the epiderms. Our assay utilizes green fluorescent protein (GFP)+ keratinocytes and GFP- keratinocytes from the interfollicular epidermis of neonatal GFP transgenic mice. One million GFP- keratinocytes are seeded with variable numbers of GFP+ keratinocytes in a silicone chamber on the fascia of a severe combined immunodeficient mouse(SCID), and produce an epidermis in two weeks. Epidermis is harvested at 5-6 weeks, ensuring that all transit-amplifying and differentiated cells have been lost. Chamber epidermis thus consists of stem cells initially added, along with their subsequent progeny. Such long-term competitive assays ensure the detection of only the most primitive stem cells. A limiting dilution design allows stem cell quantification. To determine stem cell frequency in neonatal murine epidermis limiting dilutions of GFP+ keratinocytes were implanted, ranging from 50,000 to 3,250 GFP+ keratinocytes. At 5-6 weeks presence or absence of a GFP+ epidermal proliferation unit was determined by fluorescent microscopy of epidermal whole mounts. Two different statistical methods for limiting dilution analysis, L-Calc and GEN-MOD, were used. Stem cell frequency of neonatal murine epidermis was 1 in 26,500 (95% confidence limits (1 in 14,000 to 1 in 51,000). The p-value of $\chi 2$ was not statistically significant, demonstrating the validity and lack of inconsistency in our assay. This assay will also allow us to study changes in stem cell frequency with age and disease and to determine the efficacy of various putative stem cell isolation procedures.

0528

Consequences of dominant-negative mutations in serine palmitoyltransferase: hereditary sensory neuropathy and reduced epidermal barrier integrity

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Ceramides (Cer) are critical lipid components of the epidermal permeability barrier. Serine palmitoyltransferase (SPT), the enzyme that catalyzes the first committed step in Cer synthesis, comprises a heterodimer of two subunits derived from separate genes, SPTLC1 & SPTLC2. Mutations in SPTLC1 are associated with hereditary sensory neuropathy type 1 (HSN1), a dominantly-inherited degenerative disorder of peripheral nerves (Bejaoui, Nat Genetics 27:261, 2001 & JCI 110:1301, 2002). We investigated effects of SPTLC1 mutations (C133Y & C133W) identified in two HSN1 families, on extracutaneous SPT activity and sphingolipid synthesis, stratum corneum Cer distribution, ultrastructure, and epidermal barrier integrity. Although neither C133Y & C133W mutations altered steady-state protein levels for SPT1 (or SPT2), each reduced SPT activity and Cer synthesis (approx 50%) in HSN1 lymphoblasts. Consistent with a dominant-negative mechanism, mutated SPT1 impaired the ability of normal SPT1 subunit to complement the SPT deficiency in Chinese hamster ovary (CHO) cells lacking normal SPT1, and inhibited SPT activity in normal CHO cells. Baseline transepidermal water loss (TEWL) was elevated (1.5- to 2.0-fold), and barrier integrity was compromised (TEWL >2.5-fold elevated after 10 consecutive D-Squame strips) in an HSN1 patient with advanced peripheral neuropathy compared with healthy control values. Despite diminished barrier function, ultrastructure revealed areas with normal-appearing stratum corneum lamellar membrane structures, and epidermis displayed a typical distribution of Cer species. These studies reveal a dominant-negative mechanism for SPT mutations associated with HSN1, and suggest, despite altered SPT1 function, near-normal baseline epidermal de novo Cer generation, possibly reflecting extra-cutaneous and/or dietary compensation.

0529

Calcium-fluorescence lifetime imaging in *ex vivo* skin

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Calcium controls essential processes of epidermis and keratinocytes, such as differentiation, signaling, and cell adhesion, and is important in a number of pathogenetic processes, e.g., Darier Disease, Hailey-Hailey Disease, and Psoriasis. Previous studies of Ca^{2+} signaling in epidermis using electron microscopy with Ca^{2+}-precipitation methods, by proton-induced X-ray emission (PIXE), or by applying Ca^{2+}-sensitive dyes to tissue sections were limited, in that these technologies can determine Ca^{2+} in only very small sample volumes, require frozen or fixed tissue, or determine total Ca^{2+} only, irrespective of ionization- or binding-state. Only in cultured cells have fluorescent dyes and transfected constructs been used to assess cellular or subcellular Ca^{2+} concentrations. Thus, extracellular and intracellular absolute Ca^{2+} concentrations are unknown. In our recent publications, we described Fluorescence Lifetime Imaging Microscopy (FLIM) to assess pH in intact epidermis. Here we report another application of FLIM, to measure and visualize Ca^{2+} in ex-vivo biopsies of unfixed epidermis. We show overall increasing Ca^{2+} concentrations from basal (SB) to granular (SG) layers, confirming our prior PIXE and Ca^{2+}-precipitation results. In addition, FLIM demonstrates a shift in the cellular Ca^{2+} distribution pattern: while in the SC highest concentrations seem to be limited to the cellular domain, i.e. the corneocytes, within the SG highest Ca^{2+} concentrations are found in the extracellular compartment and in an ER-like distribution intracellularly, while the cytosol itself displays very low Ca^{2+} concentrations. Currently, we are expanding the dynamic range of the FLIM methodology, so that both intracellular changes in the nanomolar range and extracellular changes in the micromolar range of Ca^{2+} concentrations can be assessed. Comparison of changes in these two compartments and their regulation are especially important to assess functional consequences in barrier homeostasis and repair, as well as various pathologic states.

0530

Differentiation-dependent transcriptional regulation of serine palmitoyltransferase in human keratinocytes

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Serine palmitoyltransferase (SPT), the rate limiting enzyme for sphingolipid synthesis, is a heterodimer of two distinct gene products, SPT1 and SPT2. Given the importance of epidermal sphingolipids, including ceramide (Cer), glucosylceramide (GlcCer) & sphingomyelin as barrier lipid components, we are studying the mechanisms by which SPT activity is regulated during epidermal differentiation. We previously demonstrated that both SPT and GlcCer synthase expression are increased through a PKC-dependent mechanism in cultured human keratinocytes (CHK) during tetradecanoyl phorbol 13-myristate (TPA)-induced differentiation. Consistent with SPT activation, Western blot analyses now reveal increases in both SPT1 & SPT2 subunits following TPA (10 nM) treatment. Although quantitative real-time PCR analyses demonstrate a significant increase in SPT2 (2.3kbp) mRNA with time (1.6-, 3.0- & 1.8-fold at 8, 16 & 24h, respectively) following TPA, SPT1 mRNA was not significantly altered at any time point. Parallel Northern blot analyses confirmed the increase in SPT2 mRNA, with no alteration in SPT1 mRNA levels. The pan-PKC inhibitor (Bis-IM) abolished the increases in both enzyme activity and SPT2 mRNA levels, confirming the PKC-dependence of this response. Finally, TPA significantly increased SPT2 promoter activity (4-fold; $p<0.01$) in CHK transfected with a luciferase construct containing 1.8kb of the human SPT2 upstream promoter. Given that SPT1 heterodimerizes with SPT2 to form active holoenzyme, these results: 1) demonstrate that transcriptional upregulation of SPT2 is responsible at least in part for PKC-dependent upregulation of SPT activity; 2) reveal that post-transcriptional regulation of SPT1 protein levels also contribute to differentiation-dependent increases in SPT activity; and 3) suggest that constitutive SPT1 mRNA levels are sufficient to accommodate differentiation-dependent increases in SPT activity and subsequent sphingolipid synthesis.

0531

Linoleate is required for normal omega-esterification of ceramide & corneocyte lipid envelope formation: insights from murine essential fatty acid-deficient epidermis

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Omega-hydroxy (OH) ceramides (Cer) are unique to the stratum corneum and essential for epidermal permeability barrier homeostasis. Whereas acylated-omega-OH-Cer (acylCer), esterified predominantly with linoleic acid, serve as constituents of extracellular lamellar bilayers in the SC, non-esterified omega-OH Cer are covalently bound to cornified envelope proteins to form the cornified-bound lipid envelope (CLE). Our prior studies reveal the following sequence for the formation of omega-OH Cer & the CLE: 1) very long chain fatty acid synthesis precedes omega-hydroxylation and subsequent Cer formation; 2) acylCer formation precedes acylGlcCer generation; 3) glucosylation of omega-OH Cer and/or acylCer precedes covalent attachment to the CE; and finally, 4) deglucosylation of acylGlcCer & covalently-bound omega-OH GlcCer is the final step that generates acylCer of the extracellular lamellar bilayers and omega-OH Cer of the mature CLE. Here, we used the essential fatty acid-deficient (EFAD) murine model to investigate a key intermediate step; i.e., whether omega-esterification, specifically with the essential fatty acid, linoleate, is required for normal acylCer and/or acylGlcCer formation. A significant decrease in the levels of both (unbound) acylGlcCer (0.84 vs. 2.1 ug/mg dry epidermis; $p<0.01$) and covalently-bound omega-OH Cer (0.82 vs. 1.9 ug/mg dry epidermis; $p<0.001$) was evident in EFAD vs. normal epidermis, respectively. Although the content of both non-linoleate-containing acylCer and other unbound Cer species were largely unchanged, the levels of GlcCer were significantly increased (>25%; $p<0.01$) in EFAD vs. normal epidermis, confirming that glucosylation of normal acylCer precedes, and may be required for, normal CLE formation. The diminished acylGlcCer and CLE despite near-normal acylCer levels indicate that non-linoleic-containing acylCer are inefficient substrates not only for glucosylation, but also for CLE formation.

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Is the filaggrin-histidine-urocanic acid pathway required for stratum corneum acidification?

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Until recently, little was known about the origin or function of the stratum corneum (SC) acid mantle, beyond its putative link to colonization by normal and pathogenic microbial flora. Recent studies point to additional key SC functions that are regulated by pH: barrier function, SC integrity/cohesion, and cytokine activation/release. Although exogenous processes, such as microbial metabolites, sebaceous lipids, and/or eccrine gland products have been proposed to account for SC acidification, 2 endogenous processes recently were shown to mediate SC acidification: the sodium-protein (NHE1) antiporter and phospholipid-derived free fatty acids. In this study, we examined the importance of urocanic acid (UCA) generation by histidase-catalyzed deamination of histidine for SC acidification. To assess the role of this pathway, we measured SC pH, hydration, and permeability barrier homeostasis in 2 murine models, Peruvian (his/his mutant) mice, which display <10% normal histidase activity, and in spontaneous mutant 'flaky tail' mice that reduced filaggrin, the precursor of most, if not all of the histidine in the SC. Homozygous animals from both models displayed a normal SC pH, normal SC hydration, and normal barrier function (both basal & recovery kinetics). Further, normal neonatal animals showed no increase in SC histidase activity during post-natal acidification of the SC, suggesting that this pathway does not contribute to development of the SC acid mantle. Together, these results suggest that the histidase pathway is not required for normal SC acidification.

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Effects of multi-lamellar emulsion (MLE) on glucocorticoid induced barrier impairment

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Repeated treatment with topical glucocorticoids can result in cutaneous abnormalities. The abnormality in barrier homeostasis is corrected by ceramides, cholesterol and free fatty acids, which suggests that the glucocorticoid induced inhibition of epidermal lipid synthesis is a major factor accounting for the derangement in cutaneous function. In a previous study, the combination of MLE and steroids was shown to have a preventive effect on glucocorticoid-induced skin atrophy. In this study, the levels of the mRNA for HMG-CoA reductase, fatty acid synthase (FAS), serine palmitoyltransferase (SPT) following 3, 6 days of topical 0.05% clobetasol-17-propionate (CP) and 0.05% CP in MLE (MLE/CP) treatment were measured using a quantitative real-time PCR system. In addition, this study examined whether providing MLE, as a lipid mixture twice daily for a week, immediately after CP treatment would further normalize the glucocorticoid-induced barrier impairment. The results of the first study showed that the MLE/CP treated skin had a slightly smaller reduction in the HMG-CoA reductase, FAS and SPT mRNA levels compared to the CP treated skin. The results of the second study showed that the topical application of CP to intact skin resulted in a significant increase in the TEWL values compared to those from the vehicle treated group after a week ($p<0.05$), whereas the topical application of MLE immediately after CP treatment prevented the glucocorticoid-induced TEWL increase. As for the epidermal thickness measurements using light microscopy, the MLE treated skin immediately after CP treatment for a week showed a slightly lower decline than the CP treated skin. In addition, the EM findings showed relatively well-conserved lamellar bilayers in the MLE treated skin compared to the CP treated skin. These results suggest that the application of MLE after a CP treatment should be effective in restoring the barrier impairment induced by glucocorticoids.

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Up-regulation of Tight Junction proteins in skin with altered barrier function

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Tight Junctions (TJ) are cell-cell junctions which are long known to play an important role in the barrier function of simple epithelia and endothelia. Recently we and others have shown the existence of TJ in stratified epithelia e.g. the epidermis. Knock-out mice deficient in claudin-1, which is an important TJ-transmembrane protein, die at the first day after birth due to a tremendous transepidermal water loss, hinting for the importance of TJ also for the barrier function of the skin. We were interested in the expression and synthesis of TJ proteins in human and mouse skin with altered barrier function, e.g. due to diseases, tape-stripping or culture conditions. By using RT-PCR-techniques, immunolocalization and cell culture techniques we can show the time-dependent upregulation of various TJ proteins, e.g. occludin and claudin 4, after impairment of the barrier function of the skin. Moreover we show a down-regultation of TJ proteins in areas of diseased skin with acute inflammation. The putative role of the various TJ proteins in the barrier function of the epidermis is discussed.

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Expression of neutrophil gelatinase-associated lipocalin gene in human keratinocytes

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Neutrophil gelatinase-associated lipocalin (NGAL) was originally identified as a 25-KDa protein covalently associated with 92-KDa gelatinase from human neutrophils. Besides neutrophils, it is now known to be expressed in most tissues normally exposed to microorganisms and its synthesis to be induced in epithelial cells during inflammation. However, there have been no studies about its expression in the epidermis. We isolated NGAL gene using suppression subtractive hybridization to identify differentially expressed genes during keratinocyte differentiation. Northern analysis found that its mRNA expression was increased in response to high concentrations of calcium in cultured human keratinocytes. In situ hybridization and immunohistochemistry in normal human skin also confirmed its expression in human epidermis. Westhern blot analysis showed abundant NGAL protein during calcium-induced differentiation of human keratinocytes. Because NGAL protein is a secretary protein, a specific enzyme-linked immunosorbent assay was also used for the determination of NGAL in cultured human keratinocytes medium, showing that the secretion of NGAL was increased hundreds times in a time-dependent fashion following the addition of calcium in the medium. Based on the these observations, there may be suggested that NGAL was expressed in human epidermis and its synthesis was induced during keratinocyte differentiation, but further studies are necessary in order to determine the functional roles of NGAL in human skin biology

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A novel approach for the analysis of the nerve end organ in human skin

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Bearing the sensory nerve end organ, the skin is establishing contact to the environment and is as such a very important part of our nervous system. Nevertheless, most of our knowledge about the architecture of the skins nervous system is originating from the middle of the 20th century and still surprisingly little is known about its three-dimensional topography. We used dermal sheet preparations, immunohistochemical staining thereof with antibodies to PgP9.5 and NCAM/CD56 and subsequent confocal laser scanning microscopy in order to analyse the intact nerve end organ in situ in three dimensions. Both antibodies showed a regular network of fibers throughout the entire superficial dermis. Staining for PgP9.5 was weaker and nerve fibers were not decorated continuously, but in a bead like fashion. NCAM/CD56 staining additionally presented protrusions with diameters ranging from 5 to 15µm. In further immuno-electron microscopic analysis, we detected evidence for ultrastructural features of Schwann cells ensheating terminal nerve endings. These endings appeared to end freely and spread out within 25µm below the dermo-epidermal junction. To visualize the topographic relationship of the nerve end organ with other skin components, double staining with NCAM/CD56 and anti-vWF was done. Larger trunks of the nerve end organ were found to intertwine with the superficial capillary plexus but no obvious relation of terminal nerve endings and blood vessels could be found. Our novel approach will help to further characterize the nerve end organ under physiologic conditions as well as during diseases of the skin. The fact, that nearly all penicillate nerve endings spread out below the dermo-epidermal junction should make us think again about the presence of nerve fibers in the epidermis.

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***In vitro* assessment of cytoskeletal instability in patients with various keratin mutations**

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The cytoskeleton of keratinocytes is composed of keratins, which constitute the intermediate filaments (IF). Mutations in different keratin genes cause weakening of the IF network leading to various skin fragility syndromes (Epidermolysis bullosa simplex, due to K5/14 mutations and Epidermolytic hyperkeratosis, due to K1/10 mutations). Such patients constitutively express an increased level of heat-shock proteins (Hsp:s) in the epidermis, probably because the cells are continuously repaired. To further characterize this stress-induced response in various keratin disorders, keratinocytes were exposed to mild heat treatment (42 C for 30 min.) and harvested after 0-24h of post-incubation at 37 C. The mRNA and protein levels of Hsp 27 and 72 were examined by RT-PCR and Western blot and the structure of IF by immunofluorescence, using a keratin antibody. In the heat-exposed cells the IF network collapsed and the Hsp expression was subsequently elevated in a time-dependent manner. These results support the idea of a correlation between alterations in the IF network and the stress response of keratinocytes. Combining the analysis of the IF network and expression of stress proteins after mild heat-treatment in vitro, might be a useful tool to study various methods to improve the stability of keratinocytes with mutations in keratin genes.

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Oleanolic acid stimulates differentiation of epidermal keratinocytes via PPAR-alpha pathway

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Naturally occurring triterpenoids like oleanolic acid and ursolic acid are known to have anti-inflammatory and anticarcinogenic activities in some cells. However, the effect of triterpenoid on epidermal keratinocytes is not fully understood. In this study we observed that oleanolic acid stimulates differentiation of epidermal keratinocytes. The rate of formation of cornified envelope, a marker of keratinocytes terminal differentiation, and the protein required in the process, transglutaminase were found increased in human keratinocytes treated with oleanolic acid. This is a sharp contrast with ursolic acid, which does not give effect on the differentiation of keratinocytes. In addition, we found that differentiation induced by oleanolic acid is mediated by PPAR-alpha pathway. Increase in PPRE(PPAR response element) activity due to oleanolic acid was observed to be related to PPAR-alpha activity in CV-1 cells by reporter gene assay. These results suggest that oleanolic acid stimulate differentiation in epidermal keratinocytes presumably by activating PPAR-alpha.

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Small changes in either or both Ca^{2+} affinity and Ca^{2+} transport rate in sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b will result in Darier's disease

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Sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) catalyzes ATP-dependent Ca^{2+} transport from cytoplasm into ER lumen. Darier's disease (DD) is caused by mutations in the gene encoding this protein[1)] and thus believed to be the consequence of disturbance in the Ca^{2+} homeostasis in skin cells. In the Ca^{2+} transport cycle, the enzyme is activated by binding of two cytoplasmic Ca^{2+} ions to the high affinity transport sites, and then autophosphorylated by ATP to form obligatory phosphoenzyme intermediate (*E*P). During the subsequent hydrolysis of *E*P, the bound Ca^{2+} ions are released into lumen. To understand what kind of and how much of changes in the properties of SERCA2b are actually causing DD, we performed detailed kinetic analysis on the missense mutants of which Ca^{2+} transport activity was found seemingly to be nearly normal or only moderately reduced as compared with that of the wild type. These mutants were highly expressed in COS-1 cells as the wild type, and thus recovered in microsomal fraction for the functional analysis. We found that these mutants are categorized into three groups having; 1st, reduced Ca^{2+} affinity at the Ca^{2+} transport sites (by only ~3 fold or more) but nearly normal transport activity; 2nd, normal Ca^{2+} affinity but reduced rate of *E*P hydrolysis and thus of Ca^{2+} release into lumen (50-80% of that of the wild type); 3rd, both the reduced Ca^{2+} affinity and reduced Ca^{2+} release rate. It would be expected that in the 1st group, the cytoplasmic Ca^{2+} level in the skin cells is shifted to the level higher than the normal, while in the 2nd group, the rate of Ca^{2+} transport from cytoplasm into ER lumen is slowed. Results indicate that either or both reduction (even only slight or moderate reduction) in the Ca^{2+} affinity and that in the Ca^{2+} transport rate in SERCA2b will result in DD.

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Effects of ursolic acid and oleanolic acid on the skin barrier function

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Ursolic acid (UA) and Oleanolic acid (ONA), known as urson, prunol, and malol, are pentacyclic triterpenoids which naturally occur in a large number of medicinal herbs and plants. They may occur in their free acid form or as aglycones for triterpenoid saponins, which are comprised of a triterpenoid aglycone, linked to one or more sugar moieties. Recently research, which led to the identification of UA and ONA, revealed several pharmacological effects, such as antitumor, anti-inflammatory, antimicrobial and anti-hyperlipidemic could be attributed to UA and ONA. Here, we introduced the effect of UA and ONA on acute barrier disruption and normal epidermal barrier. To clarify the effects of UA and ONA on skin permeability, both flank skin of 8-12 weeks hairless mice were topically treated with samples (2mg/ml), then measured recovery rate using TEWL. The recovery rate increased in these groups at 6h more than 20% compared to vehicle (Veh) group ($p<0.05$). For verifying the effects of UA and ONA on normal epidermal barrier, hydration and TEWL were measured for 1 and 3 weeks after UA and ONA application (2mg/ml per day). We also investigated the features of epidermis and dermis using electron microscopic (EM) and light microscopic (LM) exam. Both samples increased hydration compared to Veh group from 1 week without TEWL alteration ($p<0.005$). EM finding revealed that secretion and numbers of lamellar bodies and complete formation of lipid bilayers were most prominent (ONA >UA>Veh). LM finding showed stratum corneum was slightly increased and especially epidermal thickening and flattening was observed (UA>ONA>Veh). Using Masson-trichrome and elastic fiber staining, we observed collagen thickening and elastic fiber increasing by UA and ONA treatments. This result suggested that the effects of UA and ONA related to not only skin barrier but collagen and elastic fibers. Taken together, UA and ONA can be pertinent candidates to improve barrier function and for skin therapeutic agent.

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Investigation of effective materials to increase sphingolipids in the skin

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Sphingolipids play an important role in the homeostasis and barrier function of human stratum corneum (SC). Three major lipids in mammalian SC are ceramides, cholesterol, and free fatty acids (FFA), which are organized into extracellular bilayers that serve the epidermal permeability barrier function. Prior studies have provided indirect and direct evidence for the importance of epidermal ceramides for the barrier. Ceramides are the major component of the stratum corneum, accounting for 30%-40% of stratum corneum lipids by weight, and are composed of at least seven molecular groups. In this study, we investigated the effective materials to increase sphinolipids of hairless mice skin after barrier disruption. Serine (S), phosphatidylserine (PS), vitamin C (V), S/V mixture, PS /V mixture were topically applied on the flank of mice after tape stripping. TEWL was measured before, 0, 3, 6, 12 and 24h after barrier disruption. Topical application of S, PS, V, S/V and PS/V to hairless mice improved the permeability barrier compared with vehicle application. And also S/V significantly ameliorated on epidermal permeability barrier recovery. The major epidermal and SC lipid species were separated by high performance thin-layer chromatography (HPTLC). Analysis of lipid composition by HPTLC after S/V and PS/V application showed an obvious increase in all the main components of SC lipids, which are known to constitute the permeability barrier of the skin. We measured the gene expression of Serine palmitoyltransferase(SPT) following S, PS, V, S/V and PS/V supplementation in human keratinocyte using RT-PCR. The expression level of SPT increased significantly in serine vs vehicle-treated cells. Thus, S, PS, V, S/V and PS/V induce both increased sphingolipid production and ehhancement of epidermal permeability barrier recovery.

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Lympho-epithelial Kazal-type related inhibitor is expressed in stratified epithelia, undergoes rapid proteolytic processing and shows specific defective expression in Netherton syndrome

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We recently identified SPINK5, encoding LEKTI, a multidomain predicted Kazal-type serine protease inhibitor, as the defective gene in Netherton syndrome (NS). To study the yet unknown tissue and cellular expression of LEKTI, we generated polyclonal antibodies raised against recombinant N- and C-terminal parts of the protein. Immunofluorescence confocal microscopy showed that LEKTI is strongly expressed in differentiated cultured human primary keratinocytes (HPK). Western blot analysis on HPK extracts detected at least two glycosylated precursor isoforms of approximately 125 and 145 kDa. These are rapidly processed into C-terminal proteolytic fragments of approximately 42, 65 and 68 kDa in both cell extracts and conditioned media. Immunohistochemistry studies of normal human tissues revealed strong expression of LEKTI in the epidermal granular layer, in the innermost layers of the hair follicle and in the duct of sebaceous glands. LEKTI expression was also detectable in Hassal corpuscles of the thymus and in suprabasal layers of all stratified epithelia tested. Immunohistochemistry of skin sections from 20 NS patients showed complete absence (17 patients) or very weak and discontinuous LEKTI expression (3 patients). In contrast, strong expression of LEKTI with redistribution in the upper spinous layers of the epidermis was observed in all other congenital erythrodermas and keratinising disorders examined. These results demonstrate that loss of LEKTI expression in the epidermis is a diagnostic feature for NS. They further support a role of this protein in epithelial barrier formation, and set the stage for future investigation of the specific activity of the proteolytic fragments identified.

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EpiDerm™ Full Thickness, a dermal/epidermal skin equivalent with a fully developed basement membrane

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Paracrine signaling between dermal fibroblasts (FB) and epidermal keratinocytes (KC) is believed to modulate skin responses during contact irritant or allergic reactions. Dermal FB also play an important role in photo-aging and photo-damage, wound healing and cancer progression. To enable in vitro study of these and other dermal phenomena in which FB-KC interactions are important, a full thickness skin model composed of a FB-containing dermis/KC-containing epidermis was developed. Normal human epidermal KC and dermal FB were cultured to produce highly differentiated full-thickness tissues extending wall-to-wall in cell culture inserts. Histologic examination of the tissue shows a collagen dermis populated by numerous viable FB and an epidermis consisting of stratified KC including basal, spinous, granular and stratum corneum components. Examination of tissue ultrastructure at the dermal/epidermal junction by transmission electron microscopy revealed a well-developed basement membrane. Hemidesmosomes were observed at the basal membranes of KC, with associated tonofilaments extending into the cytoplasm. Well-defined, continuous lamina densa, lamina lucida and fine anchoring filaments were present beneath the basal KC. Anchoring fibrils with characteristic striated structure connected the lamina densa to the underlying collagen matrix. Immunohistochemical analysis of basement membrane proteins was also performed on frozen tissue sections. Protein markers of hemidesmosomes (alpha6 integrin), lamina lucida (laminin 5), lamina densa (collagen IV) and anchoring fibrils (collagen VII) were specifically localized to the dermal/epidermal junction. EpiDerm-FT overcomes shortcomings of previous skin equivalent models in terms of providing a wall to wall tissue as well as appropriate in vivo-like basement membrane development. These attributes will enable more realistic in vitro studies of dermal/epidermal phenomena.

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***In situ* characterization of proteasome in human keratinocytes—protein substrate overload with cellular age**

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Because modified proteins are accumulating in aging cells, proteasome, the main structure responsible for protein turnover, is alleged to be controlling the aging process. We have investigated the role of proteasome in keratinocyte aging. We showed *[Petropoulos et al., J. Gerontol. 2000, 55A, B220-B227]* that accumulation of modified proteins occurs in aging keratinocytes, together with decrease of proteasome specific activity in cell extracts. In order to determine true proteasome activity within cells, we developed conditions for *in situ* measurement of proteasome activities in permeabilized keratinocytes. Proteasome, as well as β-galactosidase as an aging marker, were assayed *in situ* by using fluorogenic peptides or β-galactoside, respectively, in attached or suspended cells obtained from cultures or from skin epidermis. The assay was performed either in microtiter plates or in a flow cytometer. Amounts of cellular proteins were determined by protein staining in microplates, and by FITC binding in flow-cytometric experiments. We observed a drastic increase in cell size and protein content in aging keratinocytes, while proteasome activity per cell remained grossly constant. Hence proteasome specific activity (per milligram of cellular protein) is significantly decreasing with age, and the load of protein substrate might be too large. This may result in a reduction of the relative proteolytic rate, and contribute to the accumulation of oxidated and modified proteins in senescent cells. We directly investigated this point by inducing proteasome "shortage" in cell cultures, due to the constant presence of the proteasome inhibitor MG132. Preliminary results showed that cell size, protein content, and β-galactosidase activity, tend to increase in these conditions, as well as other age-related markers. Further studies are under way in our laboratory, to verify the involvement of proteasome in the aging processes via protein overload and protein modification.

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Ceramide metabolism in the aged epidermis

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Ceramides, are a major component of stratum corneum lipids and most important for skin barrier function. The aging process reduces skin function, although basal skin barrier function remains normal, barrier repair is delayed after injuries to the skin. The present study examines the activities of ceramide providing enzymes including ceramide synthase and sphingomyelinase in the epidermis of young hairless mice (< 3 months) and aged hairless mice (>18 months). In addition, we also examined the activity of catabolic acid ceramidase in the metabolic breakdown of ceramide. The ceramide synthase activity assay was performed using 14C-labeled palmitoyl-CoA as described in J Biol Chem 275: 30344-54, 2000. Acid sphingomyelinase activity, using 14C-sphingomyelin, was determined as shown in J Clin Invest 104: 1761-70, 1999. For assaying katabolic acid ceramidase, 14C-palmitoyl-sphingosine was used as substrate according Adv Exp Med Biol 477: 305-15, 2000. Our results showed a 40% reduction in ceramide synthase activity in the epidermis of senescent mice, in contrast to young mice. A 39% acid sphingomyelinase activity reduction was shown in the inner epidemis of aged controls in contrast to young controls. Acid sphingomyelinase activity was unchanged in the uppermost epidermal layers. Ceramide catabolizing acid ceramidase activity also remained unchanged. Based on this results, we correlate reduced permeability barrier repair in senescent skin with both reduced ceramide synthase activity and lower sphingomyelinase activity in the inner layers of epidermis. Acid sphingomyelinase in the outer epidermis and acid ceramidase are essential in maintaining basal skin barrier homeostasis and remain unchanged despite the aging process.

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MMP-9 expression from human keratinocytes in culture is induced by the ultraviolet-B irradiation

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Matrix metalloproteinase-9 (MMP-9), a kind of gelatinase, is known to be secreted from keratinocyte. And MMP-9 expression in the epidermis has been reportedly induced in the case of apoptosis including ultraviolet-B (UVB) irradiation. In the previous study, we showed MMP-9 induction from human keratinocyte in culture by high calcium, which induces differentiation, a kind of programmed cell death. In addition, we also showed that concomitant up regulation of MMP-9 and involucrin transcripts are probably caused by the very similar gene regulatory elements, KRE-M9 and KRE-4, in their respective promoters. In this study, we confirmed the induction of MMP-9 expression by UVB irrradiation using primary human keratinocyte in culture. The secretion of MMP-9 was increased by 30 mJ/cm2 or more dose of UVB irradiation. More than 30 mJ/cm2 of UVB irradiation also decreased the viability of keratinocytes. Furthermore, involucrin was also induced by UVB irradiation. We are now further examining the responsible gene regulatory element in the MMP-9 promoter in the case of up-regulation of MMP-9 expression after UVB irradiation.

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Identification of normal human Merkel cells in cultures derived from human glabrous and hairy skin sites

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Merkel cells represent a minor population of the epidermis. These cells are associated with nerves and may function as slowly adapting mechanoreceptors. Merkel cells can be identified by the expression of distinct keratins (K), and K20 is considered the marker of choice for Merkel cells detection. We took advantage of the human keratinocyte extraction technique with thermolysin and trypsin, to harvest epithelial cells including Merkel cells from both hairy and glabrous skin biopsies. With appropriate culture conditions, immunolabeling for K20 revealed the presence of normal human Merkel cells in primary and first subcultures (P1 and P2) of epidermal cells indicating their capacity to remain viable in culture. Furthermore, when these K20-labelled cells were observed by electron microscopy, the presence of dense-cored neurosecretory granules could be seen confirming Merkel cells identification. Occasionally, K20-expressing cell doublets were observed in primary cultures, suggesting that proliferation could occur. In conclusion, we have been able to identify normal human Merkel cells within epithelial cell cultures, and optimization of culture conditions will provide an in vitro model to further study the biology of this peculiar population of cells.

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Matrix metalloproteinase-9 expression from keratinocyte is down-regulated through KRE-M9 element by leptomycin B

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Matrix metalloproteinase (MMP)-9 (also known as gelatinase B) has been suggested to play many roles in tissue metabolism of pathophysiological conditions including tumor invasion and metastasis, wound healing, and apoptosis including ultraviolet B irradiation, lichen planus, and keratosis. In the previous study, we showed that concomitant upregulation of MMP-9 and involucrin transcripts is probably caused by the very similar gene regulatory elements, KRE-M9 and KRE-4, in their respective promoters. Recently, much attention has been paid to leptomycin B (LMB), which is a modulator for trafficking a variety of transcription factor-related proteins from nucleus to cytoplasm. In this study, we examined the effect of LMB on the MMP-9 expression from human primary keratinocytes in culture. We observed the selectively reduced secretion of MMP-9 in compariosn with MMP-2 by gelatin-zymography and the reduced MMP-9 mRNA in comparison with GAPDH by RT-PCR after the addition of LMB. These effects of LMB were observed even in the inductions of MMP-9 expression by high calcium concentration or by the addition of TGF-β, TNF-α or IL-1 α. We confirmed that KRE-M9 element in the MMP-9 promoter is responsible for this regulation using luciferase assay. In conclusion, the application of LMB and its derivatives could be a useful remedy for many pathological conditions in which MMP-9 are involved.

0549

CYP2B19 immunoreactivity is expressed in human epidermal keratinocytes and induced during differentiation *in vitro*

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The cytochrome P450 CYP2B19 is a keratinocyte-specific epoxygenase in mouse skin. CYP2B19 generates mainly epoxyeicosatrienoic (EET) acids from arachidonic acid. It is a specific, biochemical marker for differentiated keratinocytes. P450-derived EETs are generally known to mediate signals regulating cell growth and differentiation. The CYP2B19 product 14,15-EET activates transglutaminases in situ, in differentiating mouse and human epidermal keratinocytes, and promotes cornified cell envelope formation. We propose that a major function of CYP2B19-derived EETs is to regulate epidermal cornification and that a functional CYP2B19 homologue is present in human skin. Using Western immunoblotting and anti-CYP2B19 antibodies, we demonstrated that human skin contains proteins immunologically related to CYP2B19. CYP2B19 immunoreactivity was upregulated in human epidermal keratinocytes during terminal differentiation in submerged cell culture. These results were corroborated by co-culturing human epidermal keratinocytes with collagen-embedded, dermal fibroblasts. This three-dimensional skin model provides physiological conditions mimicking in vivo gene expression patterns. After lifting the skin models to the air-liquid interface, the expression of CYP2B19 immunoreactive proteins increased continuously over 14 days. We also demonstrated relatively low levels of CYP2B19 immunoreactivity in oral keratinocytes, which naturally show parakeratotic or no keratinization. CYP2B19 immunoreactivity was proportional to the extent of keratinization. In conclusion, we show evidence for a functional homologue of CYP2B19 in human skin, which may regulate epidermal cornification. The 3D skin model will be a useful tool to elucidate functions for human P450 epoxygenases during epidermal differentiation.

0550

Age-related expression of the mu-opiate receptor in normal human keratinocytes

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There is increasing evidence that neurotransmitters play a crucial role in skin physiology. Human epidermal keratinocytes mediate signals between the skin and the nervous system through regulatory neuropeptides. This include production of POMC derivatives such as beta-endorphin, a molecule which binds with high affinity to mu-opiate receptors in epidermis. This receptor was recently shown to be expressed by human epidermal keratinocytes both at the mRNA and protein levels and that its expression was observed in the entire epidermis. Until now, no published data focus on the possible modification of the mu-opiate receptor system as a function of age in human skin cells. In this study we investigated the mu-opiate receptor expression by normal human keratinocyte strains from various healthy females (20 to 70 year old) by semi-quantitative indirect immunofluorescence analysis and confocal microscopy visualization. We observed a marked decrease of mu-opiate receptors expressed by cells from older donors compared to young ones. In addition, preliminary results on beta-endorphin secretion by keratinocytes from the same individuals suggest an increasing ability of old donors to produce this molecule even if a large heterogeneity was observed between the strains. These results point out that modification of the mu-opiate receptor system could be an important parameter in epidermal skin ageing and offer a new insight in the skin neuro-regulatory processes of the elderly .

0551

Study of tight junction components in monolayer and in fully-differentiated stratified cultures of epidermal keratinocytes treated with a skin irritant

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In the epidermis, both stratum corneum and tight junctions seem to be strongly involved in the in vivo barrier function. Occludin and claudins are transmembrane proteins of the tight junction associated with plaque proteins including ZO-1. While claudin-1 is expressed in all living layers of the normal epidermis, occludin and ZO-1 are restricted to the granular layer. In monolayer autocrine cultures of keratinocytes, the expression of claudin-1 and occludin is regulated by the confluence state of the keratinocytes. Indeed, subconfluent keratinocytes express low mRNA levels for claudin-1 and occludin. Confluence induces an important increase in both expressions. For claudin-1 and occludin, mRNA expression becomes maximal at post-confluence of the culture, suggesting that the expression of tight junction components is linked to epidermal differentiation. In parallel, study of tight junction components has been also performed in vitro on fully differentiated stratified keratinocytes cultured on inert polycarbonate filter. Tight junction components are expressed in the reconstructed tissue exhibiting localization similar to the localization reported in vivo in the epidermis. Such a reconstructed epidermis has been tested previously for its barrier function regarding a known skin irritant (benzalkonium chloride) and its cell viability has been reported as decreased in a dose-dependent manner. Benzalkonium chloride is thought to alter the barrier established at the cornified layer of the tissue. In order to analyze whether the irritant also alters tight junctions in this kind of tissue, the localization of claudin-1, occludin and ZO-1 was analyzed by immunostaining after treatment with this compound. Benzalkonium chloride induces modifications on the localization of tight junction components as showed by their diffuse staining and/or relocalization. These results suggest that junctional barrier may be affected by chemicals exhibiting a strong irritant potential.

0552

Autocrine albumin synthesis in the human epidermis

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The human epidermis forms the outer most layer of the human body and is therefore responsible to form an adequate barrier to prevent water loss. The stratum corneum contains a large amount of lipids such as fatty acids, cholesterol and ceramides. Since free fatty acids have to be bound to albumin, we asked whether this protein is expressed in the human epidermis, because Rabilloud (1989) showed the presence of serum albumin in the epidermis and suggested albumin uptake from the plasma into the epidermis. Here we present for the first time DCoH/HNF-1α (dimerisation cofactor for HNF-1, hepatocyte nuclear factor-1α) transcription of a splice variant of albumin in vitro in epidermal cells and in situ in full thickness human skin, using immunofluorescence staining for albumin, DCoH and HNF-1α. In order to demonstrate co-localisation of DCoH/HNF-1α and albumin double immunofluorescence was utilised, yielding cytosolic and nuclear localisation. The data demonstrate the major localisation of the DCoH/HNF-1α and albumin expression in the suprabasal cells, whereas the transcription and protein expression is almost absent in the basal layer. The presence of both, albumin and HNF-1α was supported by western blotting in cultured human epithelial cells. The results showed that differentiated and undifferentiated keratinocytes as well as melanocytes can transcribe and synthesise albumin and HNF-1α and that the earlier documented presence of albumin in the epidermis is independent of uptake from the blood stream. Hence the human epidermis holds the capacity for autocrine albumin synthesis. Since albumin binds calcium besides fatty acids we used computer modelling for an additional investigation of a possible calcium-binding site. Here we provide evidence for the first time for a specific EF-hands binding site for calcium.

0553

Structural basis for the epidermal functional abnormalities in Vohwinkel disease

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Although loricrin is the predominant protein of the cornified envelope (CE), both k.o. and overexpression mutations produce minimal phenotypic alterations. In contrast, dominant-negative mutations in the C-terminal domain of loricrin produce the characteristic phenotype of Vohwinkel disease (VD). Despite the recent production of transgenic mice with a comparable phenotype, the pathogenesis of VD remains unknown. To ascertain the basis for the VD phenotype, we assessed epidermal structure and/or stratum corneum (SC) function in 13 affected members of a previously-genotyped kindred of VD (Maestrini et. al., 1996), as well as in 5 unaffected family members and 6 normals. In addition to palmoplantar (honeycomb) keratoderma with pseudoainhum and mild-moderate ichthyosis, affected patients often displayed sub-clinical skin fragility (+Nikolsky), with a cleavage plane at or just above the outer granular layer. SC functional studies revealed abnormal basal permeability barrier function, but accelerated repair kinetics over both acral and proximal skin sites. Despite evidence of keratinocyte fragility, increased water loss occurred predominantly via extracellular domains, and the extracellular defect was linked to discontinuities in lamellar bilayers, further attributable to an abnormal CE scaffold (in contrast, the corneocyte lipid envelope (CLE) and its constituent ceramides were normal). The primary pathogenic role of the defective CE scaffold was further underscored by the presence of a normal lamellar body (LB) secretory system, with largely unimpeded secretion of LB contents. Accelerated barrier recovery in VD was explicable by amplified LB production and secretion. These results show that the barrier abnormality in VD is due to a defective CE scaffold, resulting in increased extracellular permeability, as shown previously for another 'scaffold disorder', lamellar ichthyosis.

0554

Withdrawn.

0555

Protective effect of tocopherol gentisate on hydrogen peroxide-induced apoptosis, necrosis and intracellular oxidation of normal human keratinocytes

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Because of their external position in skin, epidermal cells are exposed to a variety of oxidative stress among which tobacco smoke, pollutants, ozone and solar radiations are the major contributors leading to skin inflammation and premature ageing. One of the most powerful natural antioxidant is alpha-tocopherol, a lipophilic cellular free radiacal scavenger. The present study investigate the protective effect of a new tocopherol derivative, tocopherol gentisate (TG), in which vitamin E is esterified in ortho and para positions by gentisic acid (2, 5 dihydroxybenzoic acid), a natural metabolite of salicilic acid. Apoptosis and necrosis were investigated by cytometry using a new double staining with the fluorescent probes JC-1 and TOTO-3, measuring respectively the mitochondrial transmembrane potential (collapsed in apototic and necrotic cells), and the plasmic membrane permeability (maintained in apoptotic and unaltered cells). For intracellular oxidation cells were loaded with the sensitive and permeant probe dichlorodihydrofluorescein diacetate, emitting a specific 529 nm-fluorescence when oxidized. Compared to non-treated control cells, populations treated for 24 h with 35 microM TG exhibited a marked reduction of apoptotic and necrotic status in favour of an unaltered one (high mitochondrial potential and preserved plasmic membrane integrity) when exposed to 800 microM H2O2. In addition, keratinocytes treated for 24 h with 3.5 to 35 microM GT harbor a decrease of intracellular oxidation when exposed to a moderate stress (200 microM H2O2). We conclude that GT protective effect on epidermal cells may take place through a reduction of reactive oxygen species generated during acute oxidative stress, thus preventing damages of cellular compartments and organites and leading to a reduction of apoptosis and necrosis.

0556

The use of an integrated multi-photon and optical coherence tomography microscope for three-dimensional imaging of engineered skin

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Three-dimensional tissue constructs are a current focus in the field of tissue engineering. As engineered tissues become increasingly complex, visualization of cell dynamics and tissue growth in these three-dimensional constructs becomes increasingly difficult due to the thickness of highly-scattering tissue samples. An integrated multi-photon microscope (MPM) and optical coherence tomography (OCT) microscope has been constructed for visualizing three-dimensional engineered tissue scaffolds and tracking the growth of engineered tissues on a micron scale. Imaging with OCT is well-suited for this purpose because of the inherent capability of the technique to image deeper into highly-scattering tissues than is possible with confocal or multi-photon microscopy. We have constructed this integrated microscope to allow simultaneous imaging of tissue structure with OCT and of tissue functionality with MPM. Human skin epithelial cells (keratinocytes) were grown over a cultured layer of human dermal fibroblasts (Cascade Biologics Inc., Portland, OR) for a total growth period of 8-12 days. OCT and MPM images of engineered skin have been captured and compared to corresponding histology, demonstrating the use of this non-invasive, high-resolution imaging instrument. This instrument has the potential to be an enabling diagnostic tool for future studies in cell-cell interactions, cell-matrix adhesions, and the effect of mechanical and pharmacological stimuli on the growth and development of engineered tissues.

0557

The armadillo protein p0071/PKP-4 differentially regulates adherens junction and desmosome assembly

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Adherens junctions and desmosomes are the major adhesive intercellular junctions in the epidermis. These distinct adhesive structures contribute to epidermal organization and function by anchoring actin and intermediate filament networks to sites of cell-cell contact. p0071/plakophilin-4 (PKP-4), a member of the armadillo protein family, is related to both the adherens junction protein p120 and to the desmosomal proteins, the plakophilins. In A431 epithelial cells, PKP-4 localized to both adherens junctions and desmosomes, suggesting that PKP-4 might function in the coordinated assembly of these intercellular junctions. In A431D cells, a subclone of A431 that lacks classical cadherins, PKP-4 localized to the cytoplasm. Significantly, upon expression of classical cadherins in A431D cells, PKP-4 was recruited to intercellular junctions. Site directed mutagenesis and yeast two hybrid experiments revealed that PKP-4 bound directly to the highly conserved juxtamembrane domain of classical cadherins, demostrating that cadherins bind and recruit PKP-4 to intercellular contacts. In addition, PKP-4 interacted with the amino-terminal region of desmoplakin. Moreover, deletion mutagenesis experiments indicated that PKP-4 interacted with the amino-terminal 164 amino acids of desmoplakin. To examine the function of PKP-4 in the assembly of intercellular contacts, an adenoviral delivery system was used to express PKP-4 in A431 cells. Exogenously expressed PKP-4 enhanced the recruitment of E-cadherin and beta-catenin to adherens junctions. However, in striking contrast to plakophilin-1, which enhances desmoplakin incorporation into desmosomes, PKP-4 caused a dramatic loss of desmoplakin from cell-cell contacts. These data suggest that PKP-4 may negatively regulate desmosome assembly, and furthermore, that PKP-4 may coordinate the molecular cross talk that is thought to occur during desmosome and adherens junction assembly.

0558

Development of the stratum corneum pH gradient: localization and mechanisms leading to emergence of optimal barrier function

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The surface pH of mammalian infants skin decreases rapidly towards adult levels in the first postnatal week. Since basal barrier function is normal at birth, the basis for the higher risk for infections, dermatitis, and percutaneous absorption of toxic agents in infants is unknown. We hypothesized that delayed acidification of the stratum corneum (SC), which would further enhance barrier competence, accounts for the functional deficits of neonates. In this report, we measured and visualized the timing and localization of the emerging acid mantle in neonatal rat SC by two photon Fluorescence Lifetime Imaging (FLIM). After retraction/involution of the periderm, emergence of an acidic pH correlated with development of the extracellular lamellar bilayer system by day 3. Although an initial wave of acidity originates within the periderm, its activity does not influence pH-dependent lipid processing; hence, barrier homeostasis remains defective. In contrast, the progressive, post-natal development of acidic microdomains in the lower SC leads to an adult pattern of pH distribution, with acidic microdomains also in the upper SC after the second post-natal day. This second wave of acidity leads to efficient processing of lipids to form the mature extracellular lamellar bilayer system. We assessed NHE1 expression as the predominant mechanism that acidifies SC, beginning in microdomains at the SG/SC interface. Maximum protein levels of NHE1 occur just prior to birth, followed by a gradual decline in expression over the first postnatal week. Thus, neonatal skin is fully equipped to generate the acid mantle at birth, but full barrier competence requires the outward spread of acidification from the SG/SC interface. Although these results define a developmental sequence that leads first to acidification and then to barrier competence, the nature of the perinatal trigger that initiates acidification remains unknown.

0559

Identification of genes encoding novel mouse calmodulin-like skin proteins

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Epidermal differentiation is tightly controlled by a calcium gradient, which increases towards the surface of the skin. During differentiation, keratinocytes express specific proteins including calcium-binding proteins, such as the calmodulin-like skin protein (CLSP). Human CLSP was recently cloned and shown to share 52% homology with the ubiquitously expressed calmodulin. Unlike calmodulin, human CLSP appears to be expressed specifically in the skin in the stratum granulosum. Human CLSP has also been shown to bind transglutaminase 3, a key calcium-dependent enzyme involved in formation of cornified cell envelopes. These studies suggest that CLSP may be involved in regulating keratinocyte differentiation. To further explore the role of CLSP in mediating calcium signaling in the skin, we have cloned the mouse homologue of human CLSP. Mouse CLSP shares 50% homology with human CLSP. RNase Protection Assays (RPA) confirmed that expression of mouse CLSP was confined to the epidermis and other stratified epithelia, e.g. tongue, esophagus and forestomach. Consistent with observations that human CLSP was only expressed in differentiated keratinocytes, mouse CLSP was expressed at a late stage of skin development. Interestingly, analysis of the mouse genomic sequence revealed the existence of another calmodulin-like gene adjacent to mouse CLSP on chromosome 13. Subsequently, we cloned this gene and discovered that it was also specifically expressed in the epidermis and other stratified epithelia. A comparison of mouse and human genomic sequences confirmed that the region on human chromosome 10, which is syntenic with mouse chromosome 13, encodes human CLSP, as well as the calmodulin-like protein (CLP/CALML3). Future studies, including conditional knockout mouse models, will further elucidate the function of these calmodulin-like proteins in skin development and differentiation.

0560

Correlation in AQP3, CD44 and claudin-1 expression in human skin epidermis

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AQP3, CD44 and claudin-1 are three keratinocyte plasma membrane proteins. CD44 is a receptor for hyaluronate, the major component of the extracellular matrix, AQP3 is a water channel and glycerol transporter and claudin-1 is a tight junction protein. In the present study, AQP3, CD44 and claudin-1 expression were studied by semi-quantitative indirect immunofluorescence analysis in skin biospsies from 30 healthy females donors. All exhibited normal skin epidermis structure, low TEWL values (2-10 g/h/m2) and relatively high levels of all three markers. Nevertheless, results show significant differences (2 to 3 fold) in the expression of AQP3, CD44 and claudin-1 between donors. Interestingly, the expression of these markers was strongly correlated, with AQP3 and claudin-1 expression being positively correlated, while AQP3 and claudin-1 expression were negatively correlated with CD44 expression. CD44 is often found at open intercellular surfaces, thus suggesting that CD44 is associated with intercellular fluid maintenance related to hyaluronate homeostasis. The correlation between AQP3 and claudin-1 expression is consistent with a role for AQP3 in epidermis water homeostasis: the more impermeable junctions are, the more AQP3 facilitates transcellular water transport. These results suggest that regulations of these protein expressions may occur in a coordinate manner in skin epidermis without affecting epidermis structure or barrier function.

0561

Epithelial reprogramming of adult mouse central cornea to hair follicle and sebaceous glands under inductive dermal influences: a novel approach to cellular therapy

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Gene therapy in the skin has been hampered by the inability to target/identify a stem cell, and the lack of sustained gene expression. Several lines of evidence about the plasticity of stem cells in general have prompted us to consider a novel approach to the treatment of inherited skin disorders. Rather than searching for markers of the epidermal stem cell itself, we aimed at reprogramming other epithelia into skin under the appropriate inductive (dermal) influences in vivo. If the donor cells, such as cornea, were taken from an immunologically-compatible individual or did not elicit an immune response, such cells could overcome the two major obstacles in gene therapy approaches: gene introduction and targeting the stem cell. Building on the previous work of Ferarris et al, we performed tissue recombination experiments using embryonic mouse dermis from day 14.5 embryos combined with adult mouse central cornea cells. Recombinant grafts were implanted beneath the kidney capsule of normal mice and retrieved after two weeks. Fully formed pilosebaceous units with mature hair shafts and sebaceous glands had been induced, containing reprogrammed central cornea cells as their epithelial component. Thus, we have demonstrated that differentiated adult cells can regenerate epidermis under the appropriate dermal influences. In this approach to cellular therapy, ectopic donor epithelial cells, by definition, would contain an intact gene-of-interest, and importantly, it is predicted that epidermal stem cells would be sequestered during the induction of the new skin and hair follicle, thus providing a lifelong supply of genetically corrected cells. We are currently expanding this technology to include other sources of donor epithelium, as well as developing methodology to recapitulate the molecular events of epithelial reprogramming in vitro.

0562

Two mouse models for defects in epidermal barrier function reveal the existence of multiple compensatory pathways

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We have recently demonstrated that a defect in barrier function resulting from the loss of loricrin, a major cornified envelope (CE) protein, induces a compensatory response that activates the expression of known and novel CE components. Surprisingly, this occurs already in utero. In an attempt to better understand the molecular mechanisms that re-establish skin barrier function, we have compared two mouse models with different defects in the epidermal water bar" or "mortar" components of the stratum corneum. The loricrin knockout mouse represents a defect in the "bricks" component, i.e. cornified cell envelopes. The mouse model with a defect in the "mortar" component (lipids) was unexpectedly generated when we inhibited retinoid signaling by over-expressing a dominant-negative retinoic acid receptor mutant (RARα403 in the epidermis of transgenic mice (Imakado et al., 1995, Genes and Development 9:317-329). These mice exhibited altered lipid processing and failed to form lipid multilamellar structures in the stratum corneum (Attar et al., 1997, Mol. Endocrinology 11:792-800). To determine if a similar compensatory response was activated in both the loricrin knockout and RARa403 mouse, we isolated RNA from phenotypic neonates and examined the expression of several novel genes recently identified within the epidermal differentiation complex (EDC) located on mouse chromosome 3. Distinct induction patterns were observed for these genes as part of the compensatory response to repair the barrier defect in each model. These results suggest that different signaling pathways are activated depending on whether the barrier defect is in the "bricks" or the "mortar".

0563

Manipulation of retinoid metabolism

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Vitamin A and its derivatives have been shown to provide a variety of skin benefits. Prescription retinoic acid in particular has been shown to repair photodamaged skin and provide improvements to lines/wrinkles and hyperpigmented skin. Cosmetic vitamin A derivatives, such as retinol and retinyl esters, have been reported to provide modest improvement in the appearance of photoaged skin conditions, however, are less efficacious than retinoic acid. There are several routes toward achieving increased skin benefits from cosmetically acceptable forms of Vitamin A, which include enhancing the stability and/or delivery profile of Vitamin A from a cosmetic formulation. An alternative route is to increase the efficacy via modulation of retinoid metabolism. We have identified a series of molecules that modulate retinoid metabolism and have demonstrated their effects in vitro on transglutaminase, a marker of cellular differentiation.

0564

S100A11 in human keratinocytes--evidence for multiple forms

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S100 proteins are important calcium-responsive signal transduction proteins that act by binding to target proteins to alter target protein function. Although S100 proteins are thought to be important calcium signal sensors in keratinocytes, limited information is available regarding their distribution and function. In the present report, we show that soluble S100A11 protein is present in the cytoplasm, co-localized with the S100A11 target protein, annexin I, while non-dissociable high molecular weight S100A11-containing complexes are present with the cytoskeleton. Treatment with calcium causes S100A11 and annexin I to shift to the cell periphery via a mechanism that requires intact microtubules. In contrast, S100A11 is not detected in the Golgi or endoplasmic reticulum, and calcium-dependent movement is not inhibited by Golgi or endoplasmic reticulum disrupters. Immunostaining of uninvolved and involved psoriatic epidermal tissue reveals strong cytoplasmic S100A11 staining in basal cells, and peripheral staining in suprabasal cells, but the level of staining is not increased in psoriasis. These results suggest that S100A11 exists in at least two pools, as soluble cytoplasmic protein and as part of a covalent, cytoskeleton-associated complex. As S100A11 is a known transglutaminase substrate, it is likely that the cytoskeleton-associated S100A11-containing complex is formed via the action of membrane-associated type I transglutaminase.

0565

Characterization of the novel epidermal differentiation marker suprabasin

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Suprabasin is a recently reported novel gene expressed in mouse and human differentiating keratinocytes. We identified Suprabasin using a SSH (Subtractive Suppressive Hybridization) method between the proliferative basal and differentiating suprabasal populations of the mouse epidermis. Transcripts of 0.7 and 2.2 Kb pairs were detected on Northern blots of mouse differentiated keratinocytes. During embryonic development, suprabasin mRNA was detected at day 15.5, coinciding with epidermal stratification. Suprabasin expression was also detected in the tongue, stomach, uterus and thymus. Differentiation of cultured primary epidermal keratinocytes with 0.12 mM Ca++ resulted in the induction of suprabasin. The putative translated sequence has an amino terminal domain, a central domain composed of repeats rich in glycine and alanine, and a carboxy terminal domain. Cross-linking experiments with recombinant Suprabasin indicate that it is a substrate for TGase2 and TGase3 activity. We have generated antibodies against suprabasin in hens that specifically detect the recombinant protein by western blot analysis. Immunohistochemical staining of neonatal mouse skin showed that the suprabasin protein localizes to the most differentiated epidermal layers. Biochemical properties of suprabasin will be reported. Altogether, these results indicate that suprabasin potentially plays a role in the process of epidermal differentiation.

0566

Regulation of human involucrin gene expression requires interaction of multilpe transcriptional regulatory elements and multiprotein complex formation

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Understanding the mechanisms that regulate differentiation-dependent epidermal gene expression is of the utmost importance for understanding epidermal disease. Many studies have used cell culture models, but few laboratories have studied gene regulation under in vivo conditions that permit rigorous assignment of physiological function. In the present report we describe in vivo studies that identify the DNA elements responsible suprabasal-selective involucrin gene expression. Our studies reveal that a small region within the involucrin gene promoter, the distal regulatory region, is necessary and sufficient for normal differentiation-appropriate involucrin expression. We show that this region encodes multiple, distinct DNA regulatory elements that act in a combinatorial manner to produce the complete program of differentiation-dependent expression. A first element contains juxtaposed AP1 and Sp1 sites. This element is sufficient for expression in the high granular layer, but, remarkably, lower granular layer and spinous layer expression is absent. Moreover, inactivation of the AP1 site within this element completely eliminates expression indicating that this site has a dual function - serving as an on/off switch and also directing high granular layer expression. A spatially distinct second element is required for expression in the low granular and spinous layers. This element is not able to drive transcription alone - it drives expression in the low granular and spinous layers only when linked to the first element. These results provide a concrete in vivo example of spatially distinct DNA elements cooperating to recapitulate normal differentiation-dependent gene expression. We describe a model in which proteins, anchored at DNA binding sites within elements one and two, participate in formation of a large multiprotein regulatory complex that then contacts the basal transcription machinery.

0567

Induction of aquaporin-3 (AQP3) at wound edge of mouse skin epidermis

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The aquaporin family of water channel proteins includes at least eleven mammalian homologs. In the skin, aquaporin-3 (AQP3) has been detected in the epidermis. AQP3-null mice exhibit relatively dry skin and delayed recovery after injury, suggesting the relevance of AQP3 in skin biology. The aim of the present study is to examine stress induced AQP3 protein expression. Injury was induced by incision in dorsal skin of C57BL/6J female mice. Skin tissue sections including the wound edge were isolated 6, 12, 24, 48, and 72 hrs after injury, then analyzed by immunofluorescence using an affinity-purified polyclonal anti-AQP3. AQP3 immuno-staining was detected in basal and intermediate cell layers of epidermis as well as along the external root sheath of the hair follicle and in the sebaceous gland in healthy mouse skin. AQP3 labeling was enhanced in the external root sheath of the hair follicle in the vicinity of the wound edge after 12 hrs after injury, and in the basal and intermediate cell layer of epidermis at the wound edge 48 hrs after injury, which continued through 72 hrs after the injury. The induction pattern of AQP3 after the skin injury was consistent with that of inducible keratin 17. Our studies demonstrated that AQP3 may play a role in skin recovery after injury.

0568

Ectopic expression of Klf4 accelerates formation of the epidermal permeability barrier

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Dysfunction of the epidermal permeability barrier can result in dehydration, electrolyte imbalance, and poor thermoregulation. Immature skin is a portal of entry for infectious agents and potential toxins in topically applied substances. As the skin is one of the last organs to mature in utero, premature infants born before 34 weeks gestation, are at great risk for complications. The transcription factor kruppel-like factor 4 (Klf4), as demonstrated by a targeted ablation, has a necessary and selective function in barrier acquisition. We investigated whether Klf4 is a master regulator of this process; i.e. whether Klf4 expression in utero is sufficient to establish the epidermal barrier. Specifically, we generated lines of mice that express Klf4 from a tetracycline inducible promoter when crossed with transgenic mice expressing the tetracycline transactivator tTA from the epidermal keratin 5 promoter. These mice exhibit acceleration in barrier acquisition as manifest by the exclusion of a dye solution one day earlier in development than controls. Underlying this dye impermeability are morphological changes, including an increased number of stratified layers, expression of terminal differentiation markers, and assembly of cornified envelopes. By all criteria, Klf4 ectopic expression accelerates the normal process of terminal differentiation. Although other transgenic mice have perturbed barrier acquisition, these mice are the first to selectively accelerate the process of barrier establishment. These studies show that Klf4 is a master regulator of barrier acquisition and provide an animal model for studying how to accelerate the process of barrier acquisition for the premature infant.

0569

Formation of omega-hydroxy ceramides and corneocyte lipid envelope during fetal barrier ontogenesis

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Although the stratum corneum (SC) forms between day 17 and day 18 of gestation in the fetal rat, permeability barrier function does not become competent until day 20-to-21 (term =22 days). Since omega-OH ceramide (omega-OH Cer), are essential for epidermal barrier function, we investigated the emergence of both unbound and covalently-bound (i.e., corneocyte lipid envelope or CLE) omega-OH Cer and their respective precursor, acyl-glucosylCer (acylGlcCer), in relation to barrier ontogenesis in the fetal rat model. Both acylGlcCer and other unbound GlcCer species were present in epidermis from gestational age 17 days, as well as in newborn epidermis. Since the levels (per mg of dry epidermis) of all glucosylated Cer species were higher in fetal than in neonatal epidermis, conversion (deglucosylation) to corresponding non-glucosylated Cer occurs at-or-near term. Likewise, the content of unbound Cer species, including acylCer, increased progressively from day 17 to day 19, and all these Cer were lower in neonatal than in day 19 fetal epidermis. In contrast to unbound GlcCer and Cer species, covalently-bound omega-OH Cer (CLE), known to be required for normal barrier function, were not detectable in day 17 fetal epidermis, with only minimal CLE evident at day 19. In contrast, the CLE-omega-OH Cer levels were markedly elevated in newborn over fetal rat epidermis (i.e., approximately 20-fold vs. day 19, $p<0.01$). Finally, covalently-bound, glucosylated omega-OH Cer were not detected at any fetal or neonatal stage. In summary, the significant postnatal increase in CLE with concurrent decreases in both acylCer and acylGlcCer suggest that the latter represent precursor lipids required for CLE formation. In addition, the lag time between the initial generation of acylGlcCer/acylCer, and the subsequent formation of the CLE suggests not only that this latter event occurs efficiently during the final stages of epidermal barrier ontogenesis, but also that deglucosylation of glucosylated Cer occurs quickly following their covalent attachment to the cornified envelope.

0570

***In vivo* evidence for H_2O_2 - oxidation of epidermal albumin in vitiligo**

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Nowadays there is convincing in vivo and in vitro evidence for mM concentrations of epidermal hydrogen peroxide (H_2O_2) accumulation in untreated patients with vitiligo. Earlier the fluorescence excitation spectrum of epidermal tryptophan has been documented in human skin at 295nm. However, the origin of this tryptophan has escaped definition so far. Therefore the aims of this study were to identify the source for epidermal tryptophan and to follow in vivo and in vitro the effect of mM H_2O_2 on the characteristic fluorescence spectrum in normal healthy controls (n=12) and in patients with vitiligo untreated (photo-skin type III, Fitzpatrick classification) (n=8) and treated (n=8). Our in vivo results identified for the first time the single tryptophan of human albumin as one source for tryptophan fluorescence and confirmed the excitation peak at 295nm. However, in untreated patients lesional skin (n=8) we found 3 different groups based on the excitation spectra a) in the region of 280nm (n=8) b) in the region of 290nm (n=5) c) in the region of 300nm (n=6). After consequent removal of epidermal H_2O_2 with pseudocatalase (PC-KUS) the excitation spectra shifted in all 8 patients to 280nm. These results suggested oxidation of epidermal albumin. Since albumin has 17 disulfide bridges it was subjected to perfomic acid oxidation. The cysteic acid residues product was confirmed by Fourier-Transform Raman Spectroscopy yielding a major peak for the S=O stretch at $1033cm^{-1}$. This peak was also detected in the lesional skin of patients with untreated vitiligo using in vivo FT-Raman spectroscopy. Based on these results we propose that high concentrations of epidermal H_2O_2 affect albumin homeostasis in the human skin. Since this protein is vital in the binding of unsaturated fatty acids, this discovery unmasked an important new pathomechanism, which needs further investigation.

0571

The lanceolate rat phenotype is characterized by a defect in hair follicle keratinization and results from a mutation in the desmoglein 4 gene

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Desmoglein 4 is a newly identified member of the desmosomal cadherin gene family in mammals. The gene was recently cloned in the mouse, rat and human genomes, and was shown to underlie both the lanceolate mouse phenotype (lah/lah), as well as the corresponding human disorder, localized autosomal recessive hypotrichosis (LAH). LAH is characterized by a localized scalp-restricted hypotrichosis with sparse and fragile hair shafts. In mouse, the lah/lah phenotype was named due to the appearance of a lance head shaped defect in the keratinization of the hair shaft, and a profound defect in the precortical hair matrix cells was described. Here, we report identification of the rat counterpart of the lanceolate phenotype, designated lah/lah. Affected animals display hypotrichosis with fragile and broken hair shafts. Histological analysis revealed the presence of lance-head abnormalities on anagen hair shafts, as well as the presence of lance-tipped hairs emerging from the skin surface. Based on the genetic and phenotypic analysis of the lah rat, we cloned and sequenced the rat ortholog of the Dsg4 gene on rat chromosome 18, and identified a missense mutation in exon 6 of the gene that was not present in a group of unrelated, unaffected animals. This residue is highly conserved in the mouse and human genomes, suggesting that it is a functional mutation. Further, this mutation occurs in the second extracellular repeat region, immediately downstream of the missense mutation identified in the lah/lah mouse allele. Comparative histological analysis of the rat and mouse phenotypes support an integral role for dsg4 in the differentiation of hair follicle keratinocytes.

0572

Multiple keratinocyte sub-populations are involved in acute and chronic wound re-epithelialisation

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The skin is a complex multifunctional organ that provides an effective environmental barrier and the objective of cutaneous wound healing is restoration of epidermal integrity. Keratin intermediate filaments are essential elements of the epithelial cell cytoskeleton and can be used to define keratinocyte sub-populations. We have demonstrated that at least 7 different keratinocyte populations are involved in wound re-epithelialisation ($K5^+K14^+$, $K1^+K10^+$, $K1^+K10^+K6^+K16^+$, $K6^+K16^+$, $K6^+K16^+K17^+$, $K6^+K17^+$, $K5^+K14^+K6^+K17^+$). However, it remains unclear if the different keratinocyte sub-populations are altered during impaired re-epithelialisation. Thus, we investigated moist versus dry acute wounds, acute versus chronic wounds and healing versus non-healing chronic wounds. 21 subjects with punch biopsy wounds treated with dry or moisture retentive dressing had post-wounding biopsies, using a "polo" biopsy technique. A further 20 subjects with chronic venous leg ulceration (VLU) had ulcer edge biopsies. The VLU were classed as "healers", if there was greater than 40% reduction in wound surface area within 4 weeks. Single- and double- immunofluorescence and immunohistochemistry studies were undertaken with specific antibodies against keratins (K5, K6, K10, K14, K16 and K17), Ki67 and integrins (α3 and α5). The data was analysed using Chi square test. In all wound types, all keratinocyte sub-populations were present. In acute wounds treated with dry dressing, the overlying scab persisted, and re-epithelialisation was slower compared to those with moisture retentive dressing. In non-healing VLU, K10 positive keratinocytes were more evident at the VLU edge ($\chi^2 = 6.13$ and $p = 0.05$). Thus we conclude that wound re-epithelialisation is more complex than previously thought and altered rates of healing do not automatically infer changes in keratinocyte phenotype.

0573

PCD/DCoH/HNF-1α protein expression as a useful marker for differential diagnosis of leucoderma

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Previously it was shown that both mRNA and enzyme activities of 4a-pterin carbinolamine dehydratase (PCD) are present in human epidermis. It was also recognised that PCD is down regulated/deactivated by mM H_2O_2 in vitiligo. The aims of this study were to examine clinical similar leucodermas (halo-nevus, regression area in melanoma and dysplastic nevus) to explore whether these leucodermas are also initiated by H_2O_2 accumulation. Furthermore, we wanted to know whether PCD exercises its function as dimersation cofactor for the transcription factor hepatocyte nuclear factor-1 (DCoH/HNF-1) in this compartment. Therefore we utilised immunohistochemistry and immunofluorescence to follow the expression of epidermal PCD/DCoH/HNF-1α and its transcriptional target albumin in the different leucodermas compared to healthy controls. The results of this study identified for the first time the presence of HNF-1α isoform and DCoH activity in human keratinocytes and melanocytes in vitro and in situ. While PCD/DCoH/HNF-1α is almost absent in lesional vitiligo, due to mM H_2O_2 levels, it is upregulated in the halo of Sutton's disease, as well as in the regression zones of melanoma and dysplastic nevus. These results have unmasked a distinct difference in the pathogenesis of these leucodermas compared to vitiligo because the involvement of H_2O_2 in the depigmentation process can be ruled out in halo nevus and the regression area of dysplastic nevus and melanoma.

0574

***In vitro* percutaneous absorption in the MatTek EPI-606X skin construct and human skin**

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The rates of absorption of chemicals in human skin can be modeled in the *in vitro* percutaneous absorption assay. Moreover, the impact of various vehicles on the permeation rates of chemicals can also be evaluated. Benzoic acid (BA), was prepared in two vehicles (acetone and petrolatum) and was tested in duplicate trials in an engineered human skin construct (EPI-606X, MatTek Corp.) and in *ex vivo* human skin. The methods used are based upon guidance by the US Food and Drug Administration, with the exception that absorption was evaluated at 3 hours after test material application. Tissues were mounted in flow-through diffusion cells (0.64 cm^2 area) and tested for barrier function by ^3H_2O passage, followed by a finite dose (9 µL) of ^{14}C-labeled BA applied at 4 µg/cm^2. Barrier function was tested by applying 200 µL of ^3H_2O onto each tissue for 20 minutes. Non-absorbed ^3H_2O was removed after 20 minutes, and the tissues were maintained in the diffusion cells for an additional 60 minutes. The amount of ^3H_2O absorbed into the receptor fluid was measured during the 80-minute test. Results of the barrier tests showed that 1.71±0.33% (n=16) and 0.36±0.13% (n=6) of the applied ^3H_2O dose permeated through the engineered and human donor skin, respectively. Results of the percutaneous absorption of BA showed that in both skin models, the permeation rates for BA prepared in petrolatum were significantly higher than for BA prepared in acetone. At 3 hours, permeation rates for BA prepared in petrolatum were 80.9% and 51.9% of applied dose, for engineered skin and human skin, respectively (1.6 fold higher in engineered skin vs. human skin). Permeation rates for BA prepared in acetone were 38.1% and 12.5% of applied dose, for engineered skin and human skin, respectively (3.0 fold higher in engineered skin vs. human skin). These results show that both the engineered and human skin can be used to evaluate the impact of vehicles on the permeation rates of test chemicals.

0575

Evidence for caspase activity during normal epidermal differentiation and in UV irradiatad skin

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Caspases are conserved cysteinyl aspartate specific proteases that, when activated, cleave substrates that result in the apoptotic phenotype. 14 caspase family members have been described, although not all tissues express the full suite of caspases. The epidermis expresses caspase -2, -3, -6, -8, -9, and -14. Caspase-14 is unique among caspases in that its expression is limited to stratifying, cornifying epithelium, while caspases-2, -3, -6, -8, and -9 are expressed in multiple tissues. The mechanism(s) of caspase interaction and activation during normal epidermal differentiation are not yet known. To investigate this question, we incubated human skin biopsies with fluorescently labeled caspase peptide substrates to localize caspase activity. A pan-caspase substrate, a caspase-3 selective substrate and a caspase-9 selective substrate were utilized. Normal skin biopsies and a biopsy from UVB irradiated skin that demonstrated erythema were examined. In normal skin, we detected caspase activity in the cells of the superficial granular layer and in a few scattered cells in the spinous and basal layers. In skin exposed to 125mj UVB, the granular layer cell caspase activity was increased in intensity, and there were increased numbers of cells in the spinous and basal layers that recognized the caspase-3 and caspase-9 substrates. We postulate that normal epidermal differentiation involves caspase activity at the superficial granular layer, while epidermal injury, as seen with UV irradiation, results in increased caspase activity in the spinous layer not seen in normal epidermis. In vivo assays using specific labeled caspase substrates will help us determine the pathways of caspase activation during normal epidermal differentiation, in diseased skin, and during the wound healing process.

0576

Caspase-14 processing is inhibited by calpain, but not caspase, inhibitors *in vivo*

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Caspase-14 is the newest member identified in the caspase family of cysteinyl aspartate-specific proteases, and contains all the structural features found in caspase family members. Caspase-14 is expressed in stratified, cornified epithelium, and is processed to its large and small subunits only in conditions that simulate normal epidermal terminal differentiation. Another feature unique to caspase-14 is that it is processed to its large and small subunits at an isoleucine/lysine site, whereas other caspase are processed at an aspartate residue (Chien et al; Biochem Biophys Res Comm 296: 911, 2002). The unique processing site of caspase-14 combined with the PEST sequences (proline, glutamate, serine, and threonine enriched regions) at the site suggests calpain may be a candidate enzyme responsible for caspase-14 cleavage. Calpain is expressed in the granular layers of the epidermis and may be activated with the increasing intracellular calcium concentrations found during epidermal differentiation. To address this question, we subjected normal foreskin organ cultures to varying amounts of the calpain inhibitor, Z-LLY-FMK, as well as to varying amounts of the pancaspase inhibitor Z-VAD-FMK. Processing of caspase-14 to its large and small subunits was inhibited in the presence of the calpain inhibitor, but was unaffected in the presence of the pan-caspase inhibitor. The novel processing site of caspase-14 and the inhibition of processing by a calpain inhibitor suggest that this enzyme has unique mechanisms of regulation during epidermal terminal differentiation.

0577

Bone marrow stem cell plasticity: assessing the ability of bone marrow stem cells to differentiate into keratinocytes

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Adult stem cell plasticity raises the possibility that bone marrow-derived stem cells may be a source for keratinocyte progenitors and be manipulated for gene therapy purposes. Previous reports describe how bone marrow-derived stem cells may differentiate into neuronal, cardiac, muscle, and liver cells in vivo, although the extent of this plasticity or transdifferentiation remains controversial. We have previously investigated the degree of bone marrow derived keratinocytes in female patients receiving bone marrow transplants from male donors. In order to further investigate differentiation of bone marrow stem cells to keratinocytes in a murine model, bone marrow from B6,129S Rosa26 mice, that constituitively express beta-galactosidase in all cells, was transplanted via tail vein injection into lethally irradiated (9.5 Gy) congenic mice (B6,129S F2/J). In tissues of transplanted mice, cells derived from donor Rosa26 bone marrow can be detected by beta-galactosidase expression using double immunofluorescence (anti-beta-Gal and anti-CD45), X-Gal staining, or C12 fluorescein di-beta-D-galactopyronoside (C12FDG) imaging, along with confocal microscopy and FACS analysis. Analysis of thymus, spleen, and lymph nodes confirmed complete engraftment of Rosa26 donor bone marrow cells. Preliminary analysis of epidermal sheets from mice after bone marrow transplant (BMT) did not demonstrate any significant detection of Rosa26 donor derived keratinocytes. Since evidence suggests that stem cell plasticity is enhanced by tissue injury, at 6 months post transplant, mice underwent localized wounding by tape stripping (n=6). Epidermal sheets assessed at different time points after wounding did not demonstrate significant numbers of Rosa26-derived keratinocytes in either wounded or non-wounded skin. These data suggest that transdifferentiation of bone marrow-derived stem cells into keratinocytes is a rare event and is not enhanced by injury.

0578

Accuskin™, the Cell Sorted Skin Equivalent, as a model for active ingredient screening

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Xgene Corporation has pioneered the development of an entirely in vitro method for the growing of full thickness human skin based upon a self-assembly method, where skin cells are induced to migrate to correct positions in the reformation of tissue layers. Previously, it was widely believed that correct migration of cells to reform the tissue layers that make up more complex organs was only possible during embryonic development, but our Cell Sorted Skin Equivalent (CeSSE) method shows that tissue cultured cells can retain the ability to migrate to reform skin under the appropriate conditions. The Xgene Accuskin is an ideal testing platform for many applications. We find that changing the procedures used in production of the product can alter key characteristics of our model. Exposure of the epidermal surface to air resulted in increased epidermal thickness and development of a stratum corneum. Oil red staining showed lipid deposition correctly localized between the layers of squames. To characterize the barrier formulations of Accuskin were exposed to 1% SDS and viability was assessed by MTT assays. Exposure to air, and subsequent increased differentiation of the epidermis, resulted in increased Effective Time-50 (ET50) values. We demonstrate a prototypical active ingredient screen with retinoic acid on Accuskin. Single applications of microgram quantities of all-trans-retinoic acid were applied to the epidermal side of the 96 well format of Accuskin. These wells were harvested 120 hours post application and examined by immunohistochemistry and western blots. Our results indicate a decrease in elastin production in the dermis of the treated Accuskin wells. Accuskin should be viewed as a platform technology that can be optimized to evaluate a variety of aspects of skin morphology and biological response to test compounds and active ingredients.

0579

Estrogen activity on human skin involves interaction with the insulin-like growth factor-I signaling pathway

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Estrogens have been suggested to exhibit a profound influence on skin aging. We have previously shown that SZ95 sebocytes incubated with several hormones at levels similar to those found in postmenopausal women showed significantly lower content of neutral lipids and increased c-myc mRNA levels compared to those detected in sebocytes incubated with hormones at levels representing 20-year-old women. In a new experimental series which tested the effects of hormones as single agents, IGF-I, but not 17β-estradiol, amplified neutral and polar lipid production in SZ95 sebocytes ($p<0.001$) and proliferation of breast area skin fibroblasts from a 60-year-old female ($p<0.01$). To investigate the hypothesis of IGF-I signaling pathway involvement in estrogen effects on human skin, human skin cells maintained in steroid-free medium were first tested and found to express IGF-I receptor (IGF-IR) and estrogen receptor-α (ERα) using RT-PCR and Western blotting. 24-72 h treatment of SZ95 sebocytes and fibroblasts with 17β-estradiol enhanced IGF-I synthesis (+28% and +41%, respectively). On the other hand, cell treatment with IGF-I also enhanced synthesis of 17β-estradiol (+37% in SZ95 sebocytes and +19% in fibroblasts). IGF-IR expression was significantly reduced under IGF-I (-41 to -48%) and 17β-estradiol treatment (-37 to -48%) of SZ95 sebocytes for 48-72 h, while fibroblasts exhibited reduction of IGF-IR expression under IGF-I treatment (-47%, 48-72 h) but an initial increase under treatment with 17β-estradiol (+32% at 48 h, -15% at 72 h). ERα expression did not change under any regimen tested. In conclusion, although skin cells express ERα making them directly susceptible to estrogens, there is obviously a cross-talk between estrogen and IGF-I signaling pathways. IGF-I is playing a major role in regulating lipid synthesis in sebocytes and proliferation in fibroblasts and may, therefore, mediate the estrogen activity on normal and aged skin cells.

0580

Initial step of hapten sensitization by nickel in an *in vitro* skin assay: suppression of allergic cellular responses after application of emollient preparation

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The aim of this study was to explore the barrier function and the usefulness of an emollient preparation Ictyane for the prevention of the induction of contact allergy by the sensitizer NiSO(4) in an *in vitro* skin model. Langerhans cells (LCs) are the most potent antigen presenting cells in the skin and play a critical role in the induction of contact allergy. Research on the phenotypical and functional changes of keratinocytes and LCs were performed on skin explants undergoing chemical barrier destruction, sodium dodecyl sulfate (SDS) irritation and NiSO(4) sensitization. Immunohistochemical analysis showed that NiSO(4) applied on the skin induced the migration of LCs from the epidermis to the dermis, upregulated the expression of intercellular adhesion molecule CD54, HLA-DR antigen molecules on keratinocytes and dendritic cells, and upregulated co-stimulatory molecules CD86 on LCs in damaged skin tissue. A synergistic effect was observed when SDS and NiSO(4) were added simultaneously. However, none of these NiSO(4)-induced responses could be found in undamaged skin sample under the same experimental conditions. To evaluate the effect of the emollient preparation Ictyane, damaged skin explants were stimulated with the sensitizer NiSO(4) during 4 hours. The emollient preparation prevented the upexpression of CD54 and the migration of LCs from the epidermis to the dermis. These results suggest that this test system can be used to predict contact sensitizing ability of chemicals in an *in vitro* sensitization assay and indicate the importance of skin barrier in the inflammatory step of NiSO(4) contact sensitization. Restoring the barrier function, Ictyane has a protective effect against stimulation with a contact sensitizer.

0581

Phenotypic diversity and mutation spectrum in hypotrichosis with juvenile macular dystrophy

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Hypotrichosis with Juvenile Macular Dystrophy (HJMD) is an autosomal recessive disorder characterized by abnormal growth of scalp hair during infancy, and by the later occurrence of macular degeneration leading to blindness during the first to third decade of life. We recently demonstrated that HJMD results from mutations in CDH3. CDH3 encodes P-cadherin, which is expressed in the hair follicle and in the retinal pigment epithelium. We assessed 4 additional familes affected with HJMD for mutations in CDH3. Compound heterozygosity for a deletion (2112delG) and a nonsense mutation (L168X) in CDH3 were found to underlie HJMD in a patient of French extraction. Both mutations are predicted to result in the absence of functional P-cadherin. Affected individuals of three apparently unrelated families of Arab Israeli origin were found to carry the same homozygous missense mutation (R503H). This mutation alters a highly conserved Ca+2-binding site in the fourth extracellular domain of P-cadherin, which affects the protein conformation. Using haplotype analysis for a series of polymorphic markers encompassing the CDH3 gene, we obtained evidence suggesting a founder effect for R503H in the Arab Israeli population. We compared the clinical features of all HJMD families identified to date as carrying deleterious mutations in CDH3 and noticed significant differences in hair morphology, associated skin findings and degree of visual disability. Thus, our results firmly establish mutations in CDH3 as the cause of HJMD and demonstrate phenotypic heterogeneity in this disorder.

0582

Genome-wide view of fibroblast and endothelial differentiation

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Although reciprocal epithelial-mesenchymal interactions are essential for skin development, the diversity and functions of stromal cells in adult tissues remain incompletely understood. We have characterized genome-wide patterns of gene expression in cultured human fibroblasts and endothelial cells derived from organs and skin at different anatomical sites. Fibroblasts from each site displayed distinct and characteristic transcriptional patterns, suggesting that fibroblasts at different locations in the body should be considered distinct differentiated cell types. Groups of differentially expressed genes include those involved in extracellular matrix synthesis, cell fate determination, and cell migration. Several genes implicated in genetic diseases were expressed in fibroblasts in an anatomic pattern that paralleled the phenotypic defects. Adult fibroblasts maintained key features of Hox gene expression patterns established during embryogenesis, suggesting that Hox genes may direct topographic differentiation and underlie the detailed positional memory in fibroblasts. In contrast, the prevailing order of endothelial differentiation is dictated by the vessel size from which endothelial cells were derived. The gene expression programs characteristic of large vessel vs. microvascular endothelial cells demonstrate systematic differences in cytoskeletal mechanics, matrix interaction, and angiogenesis. The gene expression programs which specify arterial vs. venous endothelium were identified. The arterial fate could be conferred in part by the expression of Hey2, an artery-specific transcription factor; the venous fate appears to be coordinately regulated with development of left-right asymmetry during embryogenesis.

0583

Immune-mediated loss of transgene expression in skin: implications for cutaneous gene therapy

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A clearer understanding of the immune-mediated loss of transgene from cutaneous epithelium is necessary for development of effective clinical gene therapy protocols for patients who carry null mutations in the target gene. we have used retrovirus-mediated transfer of lacZ to mouse skin as a model to investigate the mechanism of immune- mediated transgene loss in skin. Transduction of C57Bl/6 mouse skin resulted in elicitation of both humoral and cellular immune responses. Antibody responses did not play a major role in the loss of transgene, however the prevalance of transgene-specific IgG2a and IgG2b subtypes suggested a Th-1 polarized response. Infiltration of the transduced skin with CD4+ and CD8+ cells and induction of transgene-specific cytotoxic T lymphocytes implied a role for T-cell mediated responses. Transduction of mice deficient in either major histocompatibility complex (MHC)-class I or -class II molecules resulted in transient transgene expression. Only in mice lacking expression of both class I and class II MHC molecules, the skin-directed transduction resulted in the persistent transgene expression. These data indicated a primary role for T cell-mediated responses in the immune-mediated loss of transgene expression. Furthermore, CD4 and CD8 T cells have overlapping roles and either population can effectively eliminate transduced cells. Therefore, long-term cutaneous gene therapy may require development of strategies to interfere with activation or function of both T cell populations.

0584

Extracellular matrix protein 1 gene mutations and protein expression in lipoid proteinosis

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The autosomal recessive disorder lipoid proteinosis (LP) results from mutations in extracellular matrix protein 1 (ECM1), a glycoprotein expressed in several tissues (including skin) and composed of two alternatively spliced isoforms, ECM1a and ECM1b, the latter lacking exon 7 of this 10-exon gene (*ECM1*). In this study, we sequenced *ECM1* and identified pathogenic mutations in 13 unrelated patients with LP. We also generated a new rabbit polyclonal antibody to the c-terminal 15 amino acids of ECM1 and correlated skin immunostaining with genotype and phenotype. We identified nine homozygous nonsense or frameshift mutations: R53X (exon3); 243delG (exon 4); 501ins C (exon6); 507delT (exon 6); R243X (exon 7); 735delTG (exon 7); 785delA (exon 7); 892delC (exon 7) and 1190insC (exon 8), as well as two new compound heterozygous mutations: W160X/F167I (exon 6) and 542insAA/R243X (exon 6/7), none of which were found in controls. Three mutations (501insC, 507delT and R243X) each occurred in two subjects. Considerable clinical differences in disease severity were seen in patients with homozygous nonsense or frameshift mutations but there was better correlation between antibody staining and LP phenotype. Specifically, in normal skin the antibody mainly labeled the basal keratinocyte layer. Labeling was slightly attenuated in relatively milder cases (including those with exon 7 mutations) whereas it was markedly reduced in the most clinically severe cases of LP. Taken with the previously documented mutations in *ECM1*, this study demonstrates that exon 6 and 7 are the most common sites for *ECM1* mutations in LP. Clinically, it appears that mutations outside exon 7 are usually associated with a slightly more severe mucocutaneous LP phenotype, but skin immunostaining with the new anti-ECM1 antibody may provide a more accurate reflection of clinical disease severity in this genodermatosis.

0585

Representational difference analysis on Sezary cells

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The molecular nature of the abnormal cells in cutaneous T cell lymphoma (CTCL) is not clearly understood. In order to isolate specific gene markers for CTCL lymphoma cells, we have studied the cancer cells of Sezary syndrome, a leukemic form of CTCL. We used representational difference analysis to compare Sezary cells' transcribed genes with that of normal T helper cells. We have identified several candidate genes during initial screening. Confirmation with reverse transcription coupled polymerase chain reaction (RT-PCR) verified that one gene, T-plastin, was present in Sezary cells but not in normal T helper cells. Rabbit polyclonal anti-T-plastin antibody detected T-plastin protein in Sezary cell but not in normal T cells by Western blot analysis. A cytoplasmic actin-bundling protein, T-plastin has been known to be absent from the hematopoietic cells and hematological malignancies previously. This raised the possibility that T-plastin can serve as a specific marker for Sezary cells. In cultured fibroblast, T-plastin has been shown to change cellular structure and increase cell motility. Previous reports indicated that Sezary cells are more mobile than the normal T helper cells from the peripheral blood. It is possible that that T-plastin aberrant expression may be contributing to the Sezary cell motility. Future experiments are in progress to define the precise role T-plastin plays in the pathogenesis of CTCL, and to use it as a diagnostic aid.

0586

Proliferating keratinocytes are putative sources of the psoriasis susceptibility related EDA$^+$ oncofoetal fibronectin

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The EDA$^+$ oncofoetal isoform of fibronectin was recently reported to be overexpressed in psoriatic uninvolved epidermis (Ting et al, 2000). It has been proposed that the abnormal presence of EDA$^+$ oncofoetal protein at the dermal-epidermal junction in the uninvolved skin may provide the psoriatic environment in which keratinocytes are in a preactivated state with regard to mitogenic signals (e.g. T cell lymphokines). To determine the possible sources of cellular fibronectin in the non-lesional psoriatic skin we aimed to investigate whether keratinocytes could produce the EDA$^+$ oncofoetal form of fibronectin. RT-PCR studies revealed that both cultured normal keratinocytes and HaCaT cells express the EDA$^+$ splice variant of fibronectin mRNA, and in HaCaT cells the EDA$^+$/EDA$^-$ transcript ratio was elevated compared to normal keratinocytes. Keratinocytes and HaCaT cells showed intracytoplasmic staining with an EDA$^+$ fibronectin-specific antibody and among the positively stained cells many showed mitosis. Using RT-PCR and Western blot analysis we showed that in synchronized HaCaT cells the amount of both total fibronectin and the EDA$^+$ isoform change with the proliferation/differentiation stage of HaCaT cells and peaks in highly proliferating cells. Observed differences in the pattern of mRNA and protein level changes suggest that not only transcriptional but also post-transcriptional mechanisms play a role in the regulation of fibronectin expression in HaCaT cells. Taken together, our results suggest that proliferating keratinocytes could be the sources of the psoriasis susceptibility related EDA$^+$ oncofoetal fibronectin.

0587

A novel 15 basepair deletion mutation in keratin 5 causes the removal of 5 amino acids from the highly conserved 2B rod domain in Weber-Cockayne epidermolysis bullosa simplex

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Epidermolysis Bullosa Simplex (EBS) is a blistering disease usually caused by missense mutations in the keratin 5 and 14 genes. Here we report a strikingly mild form of EBS (Weber–Cockayne subtype) caused by a 15 bp deletion in exon 5 of keratin 5. The proband was the only member of a 3 generation non–consanguineous family to have EBS. Genomic DNA was isolated from whole blood and PCR of all exons of K5 and K14 performed using specific primers and amplification for 35 cycles at 94oC for 1 min, 55oC for 1min, and 72oC for 1 min. Analysis of the K5 and K14 genes from the proband identified a 15bp deletion mutation in the 2B helical region of keratin 5, resulting in the deletion of 5 amino acid residues; arginine, asparagine, lysine, leucine, alanine in positions 429 – 433. The normal reading frame of the amino acid sequence was maintained, and no other mutations were detected in K5 exons 1–4 and 6–9. The screening of 50 normal controls, and remaining family members failed to result in the finding of an identical mutation. This is the first report of a non-missense mutation in the gene encoding keratin 5, which leads to the development of EBS–WC. As the amino acid reading frame is maintained, the overall disruption to the secondary structure of the final protein product is likely to be minimised. The alpha helical conformation favored by this region already accommodates an inherent 3 residue deletion present in all keratins. Hence, a 5 residue deletion may be accommodated to a certain extent within the alpha helical structure of the protein such that the disease expression is relatively mild. In conclusion, this report gives further insight into the complicated disease mechanisms that relate genotype to phenotype in EBS.

0588

A novel compound heterozygous mutation in Werner syndrome results in WRN transcript decay

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Werner syndrome (progeria adultorum, WS) is a rare autosomal-recessive progeroid disorder. The clinical phenotype mimics accelerated aging including symptoms like premature graying, alopecia and skin atrophy. Here we report a German WS patient carrying a previously reported as well as a novel nonsense mutation of the WRN gene (1396delA and 2334delAC). By quantitative rT-PCR analysis we demonstrate, that this compound heterozygous genotype leads to almost complete WRN transcript decay. This is in contrast to previously reported mutations where a loss-of-function of stable truncated WRN gene products was considered to be the primary cause of the disease.

0589

Novel and recurrent mutations in keratin K5 and K14 genes in epidermolysis bullosa simplex Dowling-Meara: implications for disease phenotype and keratin filament assembly

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Epidermolysis bullosa simplex (EBS) is a group of autosomal-dominant genetic skin disorders with an overall incidence of approximately 1:50.000. The EBS group is characterised by basal keratinocyte lysis leading to the development of intraepidermal blisters on minor mechanical trauma. According to the severity of symptoms, EBS is subdivided into three major subtypes. The most severe form is the Dowling-Meara type (EBS-DM) showing a sudden and often life-threatening onset at birth. In this study we investigate 5 familiar and 8 sporadic EBS cases of mainly German origin. We identified six new (K5:Glu168Lys, K5:Arg169Pro, K5:Glu170Lys, K14:Asn123Lys, K14:1218delG and K14:Arg417Pro) and three previously reported (K5:Glu477Lys, K14:Leu122Phe and K14:Arg125Cys) pathogenic mutations within the K5 and K14 genes. The single base pair deletion in position K14:1218G (amino acid position 406) results in frame-shift and generation of a premature termination codon 35 codons downstream of the mutation. This mutation lead to an unexpectedly mild disease phenotype despite of a predicted severely altered protein sequence in the c-terminal 2B domain of keratin 14 including the crucial RLLEGEE motif. The study discusses possible implications of the novel mutations on protein structure, keratin intermediate filament (KIF) formation and the corresponding phenotype and summarises the spectrum of mutations reported so far in EBS-DM.

0590

The mechanism of action of a dominant connexin 26 mutation on epidermal differentiation

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The connexins, gap-junction proteins, exhibit tissue-specific and overlapping expression patterns. Mutations in connexin genes cause a range of diseases, but the roles of particular connexins in the epidermal gap junction network remain unclear. As certain mutations of connexin 26 (Cx26) cause dominantly-inherited keratodermas, we attempted to disrupt the epidermal gap junction network by expressing a dominant Cx26 mutant (D66H: Vohwinkel Syndrome) under control of a K10 promoter in suprabasal epidermal keratinocytes of transgenic mice. From 4 days after birth, transgenic mice exhibited a transient, generalised epidermal scaling, with persistent zonal hyperkeratosis of the tail, ears and digits, leading to constriction bands. Evidence for genetic modifiers of the phenotype was obtained by comparing levels of transgene expression in epidermis. The thickened stratum corneum was found to overlie areas of keratinocytes with nuclear TUNEL stain, consistent with apoptosis or premature terminal differentiation. Cx26 and the related Cx30 accumulated in keratinocyte cytoplasm instead of at intercellular junctions. However, initial data have shown that transgenic epidermal keratinocytes expressing Cx26(D66H) in culture transferred Lucifer Yellow CH, indicating that they remained well-coupled by gap junctions. It is possible that the mutant protein might act by mechanisms other than disruption of the epidermal gap junction network. This has implications for the mechanism of action of mutant connexins in several human diseases.

0591

Cell death and defective protein trafficking are characteristic of skin disease-associated connexin mutations

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Distinct germline mutations within three connexin genes: Cx31, Cx30 and Cx26 underlie syndromic and non-syndromic disease hearing loss and an array of epidermal phenotypes including ectodermal dysplasia and Erythrokeratoderma variabilis (EKV). Recently, we have described a number of functional analyses investigating the cellular effects of disease-associated connexin mutants. Here, we have performed a detailed study of the functional effects on keratinocytes of a large panel of disease-associated mutant connexin proteins. After transfection of GFP-tagged connexin fusion proteins, all epidermal disease-associated proteins were primarily cytoplasmic with limited trafficking ability. In contrast, hearing loss associated mutant proteins could traffick to the plasma membrane but dye transfer studies demonstrate defective intercellular channel activity. Further studies of the skin disease-associated Cx31 mutations demonstrated a high incidence of cell death. This was not observed with wildtype or deafness/neuropathy associated mutant Cx31 proteins. By time-lapse-imaging of microinjected mutant Cx31/EGFP protein in keratinocytes, we reveal that all published EKV mutations displayed a high rate of cell death compared to cells transfected with wildtype Cx31/EGFP protein. Cell death was clearly indicated by cell blebbing, diffusion of microinjected dye into the extracellular space and nuclear uptake of propidium iodide from the media. Fluorescence Activated Cell Sorting of WT- and mutant Cx31/EGFP transfected keratinocytes confirmed a high percentage of cell death associated with only the EKV-associated mutant Cx31 proteins. Similar to these findings, Cx26- and Cx30-skin disease associated mutations also show a reduced transfection efficiency compared to WT or hearing loss only mutant protein suggestive of increased cell death. Further experimental studies will be described towards elucidating the mechanism of mutant connexin-mediated cell death in keratinocyte biology.

0592

Interferon regulatory factor 2 is associated with type 1 psoriasis

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Type 1 interferon is known to trigger flares of psoriasis. Recently, hypersensitivity to type 1 interferon signaling was found to cause a skin disease, similar to human psoriasis, in mice deficient for the transcription factor interferon regulatory factor 2 (IRF2). The human IRF2 gene is located 50 kb from a previously identified candidate psoriasis susceptibility locus on chromosome 4q (PSORS3 at D4S1535). Therefore, we tested association of psoriasis with IRF2. We generated a sample consisting of 157 families with a total of 521 individuals. Phase could be determined in 424 patient and 220 control haplotypes. Five novel microsatellite markers were developed and typed, and complemented with three known markers to yield a set of eight markers spaced within 600 kb around the IRF2 gene, three of which being located in the gene. While no statistically significant association was detected between psoriasis and the unstratified patient population, association was detected between type 1 psoriasis at two marker loci in the IRF2 gene with p-values of 0.005 and 0.009 (alleles), 0.0077 (genotypes), and 0.049 (two-locus haplotypes). Data analysis using a statistic measuring excess haplotype sharing among patients (HSS statistic) confirmed association of IRF2 with type 1 psoriasis (p-value = 0.006). We found significant evidence for a potential protective allele (p = 0.026 after Bonferroni correction for multiple testing), as well as suggestive evidence for a potential risk allele (p = 0.082 after Bonferroni correction). These data identify IRF2 as a potential susceptibility gene for psoriasis.

0593

Effective suppression of proliferation of human melanoma cells by combinatorial siRNA molecules

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Various cancers have been considered genomic diseases with the features of oncogene activation and tumor suppressor inactivation. The genomic dysfunctionalities limit the effectiveness of conventional therapy strategies and have thus forced considerable efforts for the past decade to explore alternative types of anticancer agents. Accumulating evidence indicates that chemically synthesized or in vivo expressed short interfering RNA (siRNA) specifically and effectively direct homology-dependent post-transcriptional gene silencing. In the present study, we investigated whether siRNA could suppress the proliferation of human melanoma cells through interfering oncogene activities and recovering the functions of tumor-suppressor gene. The results showed that single siRNA or combinatorial siRNAs were successfully transfected into melanoma cells with cationic lipid complexes. These siRNA molecules not only specifically knocked down their cognate targets such as bcl-2, cdk-2, mdm-2, pkc-alpha, tgf-beta1, N-ras, vegf and gfp mRNAs, but also effectively suppressed the proliferation of melanoma cells to various extents. Our results indicated that human melanoma cells preserve RNAi machinery and chemically synthesized and vector-driven siRNAs can be incorporated into intrinsic RNAi system for silencing target mRNA molecules. Our study also showed that a combination of five different siRNAs displayed additive effects in inhibiting the growth and differentiation of melanoma cells. Taken together, our intriguing findings provide important principles for predicting and selecting efficacious siRNAs as active agents for gene silencing, and suggest that silencing of tumor-causing mRNAs by siRNA may provide the foundation for developing new powerful gene drugs for the prevention and treatment of different human cancers including human melanoma.

0594

Novel SLC39A4 mutations in Japanese acrodermatitis enteropathica patients

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Acrodermatitis enteropathica (AE) is a rare autosomal recessive disease characterized by skin involvement due to defective intestinal zinc absorption. Usually the skin lesions include erythema, erosions and small blisters in perioral, perianal regions and hands and feet, which develop at the term of ablactation. The AE gene has been localized to chromosomal region 8q24.3 and subsequently the solute carrier family 39, member 4 (SLC39A4) gene has been disclosed as the AE gene. SLC39A4 mutations have been demonstrated in several AE families, and in this study we have examined two Japanese AE families for SLC39A4 mutations. The mutation detection strategy consisted of polymerase chain reaction amplification of all 12 exons and flanking intronic sequences, followed by direct nucleotide sequencing. It revealed three novel mutations, 1017ins53, which creates a premature termination codon, and two missense mutations, R95C and Q303H.

0595

Morphological and biochemical studies of human beta-mannosidosis—identification of a novel beta-mannosidase gene mutation

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Beta-mannosidosis is a rare hereditary lysosomal storage disease cased by a deficiency of the enzyme beta-mannosidase. To clarify its pathogenesis we did electron microscopic, biochemical and molecular biological investigations of a Japanease patient with beta-mannosidosis. This is the first case of beta-mannosidosis reported in Japan and the second report in which a gene mutation is identified. The biological importance of beta-mannose moieties in glycoproteins in basal keratinocytes is suggested.

0596

Regulation of cell cycle re-enter after DNA damage imposed arrest in human keratinocytes

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Damage to chromosomal DNA induces a complex cellular response designed to delay cell cycle progression until the DNA is repaired. In the event of irreparable damage the cell undergoes apoptotic cell death in order to preserve genome integrity. The product of the proto-oncogene c-Jun and the tumor suppressor protein p53 act in concert to regulate the cell fate after UV irradiation. This function of c-Jun is exerted through negative regulation of p53 association with the p21 promoter. As a result, cells lacking c-Jun exhibit prolonged p21 induction, whereas constitutive c-Jun inhibits UV-mediated p21 induction. We have found that the dominant negative isoform of the p73 gene (DNp73), is a target of c-Jun and p53 during UV response of keratinocytes. Both c-Jun and p53 are recruited in vivo on the DNp73 promoter to induce its transcription after UV stimulation of human keratinocytes. The DNp73 protein acts to downregulate p53 response by displacing it from the p21 promoter thus allowing the termination of UV imposed arrest. Cells lacking the p73 gene display a higher amount of p53 on the p21 promoter, and are less able to re-enter the cell cycle after UV. Consistently, UV treated cells in which the DNp73 transcript has been selectively knocked-out by RNA interference display a prolonged cell cycle arrest. Our results unveil a novel regulatory loop that governs the ability of the cell to make the decision between cell cycle arrest and proliferation after UV.Importantly this regulatory loop is activated only under conditions of repairable DNA damage and cells are allowed to re-enter the cell cycle and it is already put into motion when the p53 stabilization has not yet been counteracted by either the increase of MDM2 or other mechanisms. Thus, our results identify a new paradigm in the regulation of the p53/p73 cellular response to DNA damage in keratinocytes.

0597

A novel type of epidermolysis bullosa simplex with migratory circinate erythema is due to elongated tail domain of keratin 5

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Epidermolysis bullosa simplex (EBS) is an autosomal-dominant inherited blistering skin disease characterized by intraepidermal blister formation due to cytolysis of epidermal keratinocytes after minor trauma, and categorized into three main subtypes, i.e. EBS-Dowling-Meara (OMIM 131760), EBS-Ksbner (OMIM 131900), EBS-Weber-Cockayne (OMIM 131800). We report here two unrelated families in Japan (two cases) and Korea (5 cases) having patients with an unique type of EBS and a novel mutation in the keratin gene, KRT5; i.e., frameshift and delayed stop codon inconsistent with any subtype described above. The patients showed migratory circinate erythema and multiple vesicles on the circular belt-like areas affected by erythema. Electron microscopy of skin biopsies showed a reduction in the number of keratin intermediate filaments in the basal cells without tonofilament clumping. Immunohistochemical analysis showed no staining of Keratin 5 (K5) in basal cells by using monoclonal antibody C-50 against K5/8, whereas western blot analysis showed K5 peptide. We identified a novel heterozygous deletion mutation (1649delG of KRT5) in cases of both families. This deletion was predicted to produce a mutant K5 protein with a frameshift of its terminal 41 amino acids, and 35 amino acids longer than the wild type K5 protein due to a delayed termination codon. The mutant K5 protein in the lesional skin was detected by the western blotting with affinity purified polyclonal antibody against C terminal 11 peptides of elongated K5. Since the same abnormal elongated mutant K5 gene and protein were found in the independent families, the abnormal elongated keratin protein is likely to lead to an atypical clinical phenotype that has never been reported, possibly by interfering with the functional interaction between keratin and its associated protein.

0598

Ultraviolet B-induced repression of class II nuclear hormone receptors in keratinocytes

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Class II nuclear hormone receptors heterodimerize with retinoid X receptor (RXR) and bind to response elements in target genes, which regulate differentiation and lipid metabolism in keratinocytes. In the present study we evaluated whether UV irradiation alters the expression of class II nuclear hormone receptors; peroxisome proliferator activated receptors (PPAR), liver X receptors (LXR), constitutive androstane receptor (CAR) and thyroid receptors (TR). mRNA levels of PPAR-alpha and LXR-alpha were decreased by 50% 24 hours after UVB treatment of primary cultured keratinocytes. As previously reported retinoid X receptor (RXR)-alpha and vitamin D receptor (VDR) were also decreased 24 hours after UVB exposure. In contrast PPAR-delta was increased, and LXR-beta, CAR, and TRs were unchanged. PPAR-gamma was not detectable on Northern blots. Time course studies revealed that these decreases occurred as early as 6 hours after UVB exposure. In addition, mRNA levels of acetyl-CoA-oxydase, a target gene for PPAR-alpha, and ATP-binding cassette transporter (ABC)-1, a target gene for LXR-alpha, were similarly decreased after UVB exposure. In cultured keratinocytes transfected with a response element for either PPAR or LXR, we observed a marked decrease in promoter transactivation that was not rescued by ligand treatment, over-expression of the receptor, or the combination of the two. Finally, we also observed down regulation of DRIP, a transcriptional co-activator of these nuclear hormone receptors suggesting that the inhibition of gene expression by UV light is secondary to not only decreases in PPAR and LXR alpha but also to post receptor mechanisms that link the binding of transcription factors to DNA with increased transcription. The UV-induced down regulation of DRIP, PPAR-alpha, LXR-alpha results in the decreased expression of downstream target genes that could be a protective mechanism to prevent the accumulation of inflammatory lipid metabolites.

0599

Germline fumarate hydratase mutations in families with multiple cutaneous and uterine leiomyomata

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Cutaneous leiomyomata are rare benign tumors originating from the arrector pili muscle of the hair follicle. They can be associated with uterine fibroids in a syndrome called multiple cutaneous and uterine leiomyomata (MCL) and with uterine leiomyomata and renal cell cancer (HLRCC). MCL is inherited as an autosomal dominant trait, providing an excellent opportunity for the study of the common non-Mendelian manifestation of isolated uterine fibroids. Previously, linkage analysis identified the genetic locus for MCL and HRLCC on chromosome 1, and mutations were identified in the fumarate hydratase gene (FH). Fumarate hydratase, a component of the tricarboxylic acid cycle, has been hypothesized to act as a tumor suppressor gene in the development of cutaneous and uterine leiomyoma and renal cell cancer in this syndrome. Here, we report the clinical and mutational analysis of five families with MCL, with the identification of five new mutations affecting highly conserved residues of the FH protein. The identification of a particular FH mutation in each MCL family will allow for close follow-up of those individuals at risk for the development of aggressive papillary renal cell cancer. These results provide further evidence for the role of the FH gene in the pathogenesis of MCL and may eventually facilitate the development of new therapeutic options for affected individuals.

0600

The involvement of immunoglobulin E class switch in scleroderma skin tissue

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It has been suggested that of mast cells are involved in the formation of Systemic sclerosis (SSc) abnormalities. The mechanism of mast cell activation in SSc, however, remains unknown. Immunoglobulin isotype switching is an important process whereby B cells generate different types of effect on antibody molecules. We sought to determine whether switch circular DNAs are excised via looping out and deletional recombination in undergoing isotype switching in SSc. Isotype switch to the e locus can be achieved by direct switch from m to e, sequential switching from m to e via g, and triple step sequential switching from m to e via an a and g gene. We used nested PCR to amplify and analyze the S fragments from switch circles for direct and sequential switch for all the samples. We confirmed the results by sequencing the PCR products after TA cloning. Twenty-eight of 52 SSc skin tissues showed the presence of IgE class switch. This occurrence rate was significantly higher than those of normal skin tissue (4 of 62, $P<0.01$) and that of SSc blood cells (3 of 27, $P<0.01$). No significant difference was found for direct switch among SSc tissue (5), SSc blood cells (1) and normal tissue (4). The occurrence rate of sequential switch in SSc tissue (23) was significantly higher than that of 2 SSc blood cells ($P<0.01$). These results showed that IgE class switch is specifically involved in the formation of SSc skin tissue abnormality.

0601

Mild recessive epidermolytic hyperkeratosis associated with a keratin 10 donor splice site mutation in a family of Norfolk Terrier dogs

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Although the genetic basis for a large number of heritable epidermolytic diseases has been recognized in humans, a spontaneous keratin mutation in a non-human species has not been confirmed. In this report, we describe the phenotype and genotype of a mild form of epidermolytic hyperkeratosis in an inbred family of Norfolk Terriers. Seven affected dogs were identified in a family pedigree extending through 5 generations consistent with an autosomal recessive mode of inheritance. In all dogs, the disease manifested shortly after birth when drying with a towel resulted in blister formation. As animals aged, the skin became hyperpigmented and mild trauma continued to result in loss of the upper epidermis. Histologic features included mild epidermal hyperplasia, epidermolysis with separation at the granular cell layer, abundant large keratohyaline granules and a hyperkeratotic stratum corneum composed of thin corneocytes. Electron microscopy confirmed cytolysis and decreased tonofilaments. Clinical and morphologic features suggested a mutation in one of the superficial keratin genes. Immunohistochemical staining of formalin-fixed sections for keratin 10 (K10) and keratin 1 (K1) demonstrated no K10 staining in affected dogs but normal K1 staining when compared to controls. Based on these results, the genomic DNA sequence for canine K10 was generated in unaffected and affected dogs by PCR. Sequence analyses revealed affected dogs were homozygous for a G- to- T mutation at +1 position of the consensus GT donor splice site in intron 5 of K10. This mutation activates alternative exonic and intronic donor splice sites in intron 4, exon 5 and intron 5 resulting in premature termination codons in most transcripts that occur in sites corresponding to a region at the end of the 2B rod domain. The outcome of this mutation appears to be a marked decrease in K10 mRNA levels and an absence of normal protein as demonstrated by real-time PCR and Western blotting.

0602

Severely reduced levels of the mRNAs for lysyl hydroxylase 2 in skin fibroblasts from three patients with Ehlers-Danlos-like symptoms do not result from mutations in the coding region for LH2

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The Ehlers-Danlos syndrome encompasses a heterogeneous group of connective tissue disorders characterized by tissue fragility, hyperelasticity of the skin and hypermobility of the joints. Patients with the autosomal recessive type VI form of EDS (EDS VI: kyphoscoliosis type) have a similar clinical phenotype accompanied by significant kyphoscoliosis. At the biochemical level, skin fibroblasts from EDS VIA patients (the originally-described form of EDS VI), have significantly decreased levels of lysyl hydroxylase (LH) activity (<25% of control), as a result of mutations in the LH1 gene. LH hydroxylates specific lysine residues in the collagen molecule that are precursors for the formation of crosslinks which provide collagen with its tensile strength. In addition to LH1, two other isoforms of LH, LH2 and LH3, have been characterized, but no disorder has been linked to mutations in the genes for LH2 and LH3. We have measured LH activity, levels of mRNAs for the isoforms LH1, LH2, and LH3, and collagen crosslinking patterns in skin fibroblasts from 3 patients who have a mixed phenotype of EDS. Although their levels of LH activity (70-96% of control), pattern of collagen crosslinking in long term fibroblast cultures, and mRNAs for LH1 (69-111% of control) and LH3 (87-129% of control) were in the normal range, the mRNAs for LH2 were decreased to barely detectable levels (<5% of control). To determine whether this was due to mutations in the LH2 gene, the full length cDNA for LH2 was amplified by RT-PCR from the 3 cell lines, cloned and sequenced. No mutations could be detected in the LH2 coding sequences that could account for the severely decreased mRNAs for LH2. This suggests that the decreased expression of LH2 may be caused either by abnormalities in the regulatory region of the LH2 gene or by changes in other factors that may be required for the expression of LH2.

0603

Pathogenic mutations affecting Cx31 and Cx30.3 impair gap junction function and induce cell death

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Erythrokeratodermia variabilis (EKV), an autosomal dominant skin disorder with transient erythematous patches and hyperkeratosis, is genetically heterogeneous and may be caused by dominant mutations in the connexin genes *GJB3* and *GJB4*, encoding connexin 31 (Cx31) and connexin 30.3 (Cx30.3), respectively. Mutations in both genes affect either the glycine 12 residue in the amino-terminus or the transmembrane domains of these closely related proteins. We investigated a series of similar or distinct mutations involving orthologous residues in both genes to directly correlate their functional consequences. The wildtype (wt) proteins and 4 different Cx31 or Cx30.3 mutants were fused at the 3′end with autofluorescent YFP or CFP and expressed in HaCat cells in comparison to mock vector controls. Fluorescence microscopy confirmed proper protein expression and revealed formation of large gap junction plaques with wtCx31 and wt Cx30.3. The mutants F137L-Cx31 and F137L-Cx30.3 both accumulated in the cytoplasms of cells and did not form visible gap junctions. Cell-cell communication between expressing cells did not exceed background levels as shown by dye coupling. In addition, the expression of F137L-Cx31 was associated with a high incidence of cell death, while this characteristic was much less pronounced for F137L-Cx30.3. Co-expression of F137L-Cx31 with wtCx31 did not rescue the phenotype or prevent cell death, illustrating the dominant effect of this mutation. Formation of gap junction plaques was variable for other Cx31 and Cx30.3 mutants but all showed reduced to absent dye coupling. Our results demonstrate that different connexin mutations, depending on their nature and location, lead to distinct pathomechanisms yet any disturbance of the normal function of Cx31 or Cx30.3 results in disease.

0604

Detection of retroviral-mediated expression of latency-associated peptide delivered by transduced macrophages to murine skin

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We have developed a new method to deliver an immunomodulatory molecule, latency associated peptide (LAP), a naturally occurring antagonist for TGF-β to murine skin by gene transfer utilizing UV light to produce a cutaneous influx of monocyte/ macrophages. Bone marrow (BM) cells from BALB/cJ mice are transduced *in vitro* with a retroviral construct (MSCV) containing green fluorescent protein (GFP) for detection, human LAP (hLAP) and an internal ribosome entry site (IRES). The transduced BM cells are then driven *in vitro* to monocyte/ macrophages with GM-CSF. A construct containing only GFP serves as control. Four million transduced monocyte/ macrophages containing either GFP or GFP-hLAP are injected intravenously into BALB/cJ mice and then one group is exposed to UVB (72MJ) to induce monocyte/ macrophage infiltration into skin. Recipients are sacrificed 60 hours after UV irradiation. Western blot and immunostaining are used to document the synthesis of hLAP. PCR is used to detect GFP and hLAP sequences in recipient skin. By flow cytometric analysis more than 80% of transduced BM cells are driven to CD11b+ and MOMA+ monocyte/ macrophages by 10 days of culture in GM-CSF. When stained with anti-hLAP Ab, BM cells transduced with construct containing GFP-hLAP are both GFP and LAP positive. In recipient skin, we found transduced macrophages expressing GFP by examining the fresh frozen skin sections by fluorescence microscopy. We also detected GFP and hLAP sequences by PCR analysis in recipient skin in which GFP and LAP are stronger in UV irradiated skin compared with non-UV skin. GFP and LAP sequences are also detected in other organs (lung, blood, spleen) by PCR analysis. Our data suggest that human LAP gene product can be delivered to skin with overexpressing transduced cells. This macrophage-based gene transfer method may be a potentially useful immunotherapy for monocyte/ macrophage and TGF-β-driven fibrosing diseases such as scleroderma.

0605

Unique human keratinocyte cell surface gene products identified by serial analysis of gene expression (SAGE)

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Better characterization of global expression of keratinocyte (KC) membrane proteins will enable identification of novel KC specific markers. Traditional proteomic approaches such as 2D gel electrophoresis have not elucidated a complete representation of the KC membrane. In this investigation, the membrane fraction of human neonatal foreskin (n=15) KC mRNA, isolated by equilibrium density centrifugation, was used to create a SAGE library. This fraction of total mRNA ideally represents secreted and membrane associated KC proteins. The resulting library contained 2233 genes represented by 25,822 sequence tags. Each gene in the library is identified by a unique sequence tag, and the relative abundance of each gene is related to the frequency of occurrence of that tag. Of the 231 abundantly expressed genes (tag count >0.05% of total tags), 32% (n=73) encoded ribosomal proteins, 41% (n=95) were previously identified genes, and 27% (n=63) were unknown gene products. Previously identified genes included KC specific genes such as K1, K10, K5, K14, and membrane associated proteins such as desmoplakin, beta-4 integrin, and connexin 43. Importantly, this library was enriched for both known membrane associated proteins and unknown novel gene products when compared to published human epidermal SAGE libraries of total cellular proteins and a universal (multi-tissue) SAGE library. Furthermore, 9 of the unknown gene products were not present in the other epidermal libraries, and were limited to expression in <2% of over 3000 SAGE libraries, indicating these gene products were unique and specific to this KC library. In conclusion, the SAGE library derived from the membrane fraction of human epidermis enhances the retrieval of expressed membrane associated genes that are unique to human neonatal foreskin KC. Further characterization of the 9 tissue restricted unknown genes may identify human KC cell membrane markers useful for studying the biological function of different KC subpopulations.

0606

A very mild form of non-Herlitz junctional epidermolysis bullosa: type XVII collagen rescue by outsplicing of mutated exon 30

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Non-Herlitz junctional epidermolysis bullosa (JEB-nH) is a nonlethal variant of junctional epidermolysis bullosa with an autosomal recessive inheritance pattern. Pathogenic mutations have been found in the COL17A1 gene encoding type XVII collagen, a homotrimeric, hemidesmosomal transmembrane protein. Absence of type XVII collagen protein results in a clinical phenotype with generalized blistering, mild mucosal involvement, incomplete universal atrophic alopecia, pigment changes, rudimentary nails and dental abnormalities. Milder forms however do exist with limited blistering and preservation of scalp hair. In this study we investigated an adult patient with an extremely mild form of JEB-nH. Immunofluorescence microscopy of non-lesional skin showed reduced presence of the type XVII collagen endodomain and almost normal presence of the ectodomain. Sequence analysis of genomic DNA and cDNA identified two novel heterozygous mutations in COL17A1: a point mutation, 2356C->T, and a single base pair deletion, 3432delT. This latter 3432delT exon 48 mutation results in out-of-frame reading, thereby generating a premature termination codon. The 2356C->T point mutation in exon 30 also results in a premature termination codon, but here the reading frame is restored by skipping the 36 nucleotides long exon 30. This skipping of exon 30 is not found in normal keratinocyte mRNA. In cultured cells type XVII collagen was present at 15% of the normal level. Immunoblotting confirmed the 12 amino acid deletion by showing a type XVII collagen molecule running in SDS-PAGE at an approximately 1 kDa lower position. We conclude that this unusual mild form of JEB-nH is therefore the result of the in-frame deletion of exon 30, leading to 12 amino acid deletion in the COL15 domain and an at least partly functional type XVII collagen molecule. This particular type of rescue mechanism has not been observed before for type XVII collagen.

0607

Development of autologous, cell-based gene therapy using primary dermal fibroblasts

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We have previously demonstrated the feasibility of systemic, cell-based anti-angiogenic gene therapy for cancer treatment, using bio-engineered implants of immortalized mouse 3T3 fibroblasts that were retrovirally transduced to express the angiogenesis inhibitor thrombospondin-2. In this study, we investigated the feasibility of autologous cell-based gene therapy using primary dermal fibroblasts that were isolated from adult mouse skin. Primary fibroblasts were transduced with a retrovirus carrying a GFP reporter gene. The transduced fibroblasts were purified by cell sorting and were seeded onto 3-dimensional meshes consisting of a dense network of biodegradable polymer fibers. These cell-populated meshes were then implanted subcutaneously into the identical mice from which the primary fibroblasts had been previously isolated. Thirty days after autologous implantation, the histological detection of GFP-expressing fibroblasts by fluorescence microscopy confirmed the survival and the maintenance of gene expression of a number of the genetically engineered fibroblasts. These results indicate that autologous transplantation of retrovirally transduced primary dermal fibroblasts on biodegradable matrices might serve as novel strategy for cell-based gene therapy.

0608

The regulation of glucocorticoid receptor alpha and beta in cultured human keratinocytes by long-term, high concentrations of glucocorticoids

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The actions of glucocorticoid (GC) are mediated by the GC receptors (GR), which are divided into two isoforms, namely GR alpha and GR beta, by the alternative splicing. GR beta acts as an endogenous inhibitor against the native receptor GR alpha. The suppression of GR alpha, the up-regulation of GR beta and the decrease of GR alpha/ GR beta ratio have been reported in the human GC insensitive asthma and leukemia cases. GR alpha and GR beta were usually expressed in normal skin and we confirmed that the expression of GR alpha mRNA decreased in the steroid insensitive lesional skins as compared to sensitive lesional skins in cases of psoriasis vulgaris. The administration of steroids is usually performed daily for a long time in many clinical cases. That is why the long-term administration of steroids is thought to be involved in the acquisition mechanism of steroid insensitivity. In order to clarify the acquisition mechanism, we conducted the long-term culture of human keratinocyte under the high concentration of GC. We cultured human neonatal epidermal keratinocytes under the high and low concentrations of GC for 3 passages. We examined the expressions of GR alpha and GR beta mRNA at the end of each passage. The down-regulations of GR alpha and GR beta were confirmed in cultured human keratinocytes at mRNA level under the condition of high concentrations of GC. We are now investigating the expression of GR alpha and GR beta in cultured human keratinocytes at the protein level under the conditions of high and low concentrations of GC.

0609

***De novo* mutatations in monilethrix**

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Mutations in the hair keratins hHb1 and hHb6 have been recently reported to cause monilethrix, an autosomal dominant hair shaft disorder, characterized by variable degrees of hair fragility and follicular hyperkeratosis. We found 10 families with monilethrix in which the parents were not clinically affected, and sequenced the hair keratin hHb1, hHb3 and hHb6 genes in 7 patients. In 5 patients no mutations were found, while in 2 patients, we identified *de novo* germ line missense mutations, at the helix termination motif, E402K (hHb6) and E413K (hHb1). This study emphasizes the key role of hair keratins hHb1 and hHb6 in the pathogenesis of monilethrix, while also demonstrating the heterogeneity of this disease, and its high tendency for new mutations.

0610

Myelodysplastic syndrome in mice lacking IkappaB-alpha

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Newborn mice deficient for the gene coding for IkappaB-alpha, ikba, develop an increase in numbers of neutrophils in liver, spleen and bone marrow resulting in a myeloproliferative disease. According to the WHO category this hematological disorder would be best classified best as a chronic myelomonocytic leukemia (CMML) in the group of Myelodysplastic / Myeloproliferative diseases (MDS/MPD) or with an excess of blasts with more than 20 % blasts in the blood and / or bone marrow as a secondary acute myeloid leukemia. In order to analyse the mechanism responsible for the myeloproliferative disorder, we generated mice allowing complete or tissue-specific deletion of ikba, utilizing the Cre-loxP recombination system. An increase in granulocyte progenitors and changes in the expression level of Notch1 and its target gene Hes-1 are found in granulocytes isolated from these mice. The Notch ligand Jagged1, transiently expressed in fetal liver of wildtype mice at E12, remains upregulated in the liver of IkappaB-alpha-deficient mice. Importantly, mice with a specific deletion of IkappaB-aalpha in granulocytes show normal myelopoiesis, despite constitutive NF-kappaB activation. No Notch1 expression is found in granulocytes and no prolonged Jagged1 expression can be found in these mice. These results identify IkappaB-alpha as a key regulator in perinatal myelopoiesis via Jagged1 and Notch1. Moreover, this model demonstrates how a loss of control leadsto the development of myelodysplastic syndrome / myeloproliferative resulting in secondary acute myeloid leukemia.

0611

Genomic sequence analysis of PSORS1

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The most consistently replicated psoriasis susceptibility locus, PSORS1, maps to the MHC Class I region. We fine-mapped PSORS1 to the vicinity of HLA-C and CDSN by recombinant ancestral haplotype analysis (AJHG 66:1833, 2000). This region contains seven genes other than HLA-C, and several of these display highly significant allelic associations with psoriasis in multiple studies. However, the observed associations could generally be explained equally well or better by the presence of HLA-Cw6, an allele long known to be associated with psoriasis. In an effort to identify the PSORS1 gene, we have sequenced a 200 kb region spanning HLA-C and CDSN in 4 risk and 6 non-risk haplotypes. Sequence analysis identified over 2200 polymorphisms including over 230 insertion/deletions (indels), the remainder being SNPs. Cluster 26 (Cw7, B8, non-risk, T:NT = 53:64, TDT p = NS) displayed remarkable similarity to all Cw6-positive risk chromosomes from 0.5 to 128.5 kb telomeric to HLA-C, a region which includes the OTF3, TCF19, and HCR genes. Cluster 17 was previously found to confer marginal risk (Cw8, B65, T:NT = 21:10, TDT p = 0.048). If Cluster 17 is risk, there are no coding variations in HLA-C or any of the other 7 genes that are unique to risk. If Cluster 17 is non-risk, the only candidates for risk in the 200 kb region are HLA-Cw6 and a combination of 3 coding SNPs in the CDSN gene. However, this CDSN allele has been ruled out in Oriental populations (Tissue Antigens 60:77). Cluster 17 demonstrates substantial centromeric divergence from all Cw6-bearing chromosomes beginning 15.3 kb telomeric to HLA-C. The most prominent feature of this divergence was a 9.7 kb indel located centromeric to HLA-C. While our final conclusions will depend upon a better assessment of the risk associated with Cluster 17, the available sequence data favor HLA-Cw6 itself as the best candidate for PSORS1. This would be in keeping with the presence of CD8-positive T cells in the epidermis of psoriatic lesions.

0612

Type I collagen regulation of gene expression in collagen-related diseases

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Decreased or structurally abnormal type I collagen (COL1) leading to a reduction in cell proliferation, differentiation and extracellular matrix (ECM) protein synthesis results in conditions characterized by reduced mechanical strength of connective tissues, such as Ehlers-Danlos Syndrome types VIIA and VIIB, and Osteogenesis Imperfecta. Here we used an *in vitro* method to select cells with high survival and proliferative indices when cultured on limiting amounts of COL1. Through DNA array analysis, we defined a pattern of cytokine and cytokine receptor gene expression that correlated with the increased survival and proliferation. A mesenchymal cell line (HOS) was cultured on plastic substrates coated with either low or high levels of COL1. Collagen was labeled with ^{125}I to accurately measure site densities. mRNA transcripts encoding 700 cytokine-related genes were assessed by DNA array analysis. Immunofluorescence microscopy was used to visualize corresponding alterations in the cytoskeleton and β1 integrin distribution. We found enhanced expression of several genes involved in cell growth and ECM synthesis: transforming growth factor β-receptor II (TGFβRII), proliferation inducing ligand (APRIL), human homologue of a canine orphan receptor and tissue inhibitors of metalloproteinases 1 and 4. One of these, TGFβRII, mediated enhanced cell growth and attachment to COL1 when stimulated by soluble TGF-β, a TGFβRII agonist. In addition, stimulation of TGFβRII increased cell spreading and integrin clustering (per immunofluorescence), that correlated with increased cell survival. Subsequent, broader Affymetrix array analysis of HOS cells confirmed that the TGFβ-TGFβR-SMAD pathway is important when cells are adherent and growing on limiting amounts of collagen. This genomics approach permitted us to identify candidate cytokine gene families that may be involved in the pathophysiology of CRD, a first step in the development of new therapies for these debilitating conditions.

0613

Transgenic mouse models support HCR as an effector gene in the PSORS1 locus

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Genetic susceptibility for psoriasis is regulated to the greatest extent by the PSORS1 locus. Three strongly psoriasis-associated susceptibility alleles have been identified within it, namely HLA-Cw6, HCR*WWCC and CDSN*5, but strong linkage disequilibrium between them has made it difficult to distinguish their individual genetic effects (Asumalahti K et al., HMG 2002:11:589). With the aim of understanding the functional role of HCR, we have engineered transgenic mice with either a nonrisk allele of HCR or the HCR*WWCC risk allele under the control of the K14 promoter. These choices were motivated by the apparently dominant effect of PSORS1 on psoriasis susceptibility and the physiological expression of HCR in basal keratinocytes. The HCR*WWCC protein has been predicted to differ from the nonrisk alleles by having a shorter first alpha-helical domain. Mice that express either of the transgene mRNAs and proteins develop and grow normally. Without environmental challenge, the skin appears grossly normal for both the nonrisk and risk transgene models. Global expression studies using the Affymetrix platform revealed that five out of 12,000 genes assayed are specifically and consistently upregulated in the HCR*WWCC risk allele mice as compared to the nonrisk allele mice. These include type II epidermal keratin and two genes of the small proline-rich protein family cluster. The small proline-rich protein cluster that in human maps to the chromosome 1q21 psoriasis susceptibility locus PSORS4 is known to be among the most strongly upregulated in psoriatic as compared to normal skin (Bowcock A et al., HMG 2001:10:1793). Our results suggest that the HCR risk allele has specific functional consequences relevant to the pathogenesis of psoriasis and that HCR constitutes an essential gene in the PSORS1 locus.

0614

Hepatocyte growth factor prevents and ameliorates dermal sclerosis in a mouse model of scleroderma

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Systemic sclerosis (ssc) is a connective tissue disorder with an unknown etiology. There are currently no effective therapies for ssc. hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes, induces the mitogenic and antiapoptotic activity in various epitherial cells and prevents the organ fibrosis by inhibiting the induction of TGF-beta1. HGF may be a potent candidate to prevention of dermal sclerosis. working with a bleomycin (BLM) -induced scleroderma model mice, we performed two transfections of human HGF cDNA into the skeletal muscle and showed that this treatment not only prevented the dermal sclerosis simultaneously injected BLM but also improved dermal sclerosis induced by BLM 4 weeks before. RT-PCR, ELISA and an immunohistochemical analysis revealed that both mRNA and protein of HGF were detected inthe skin, lung and muscle and the serum after two transfections of human HGF cDNA. And these analysis also revealed that this treatment significantly reduced both the expression of the TGF-beta1 mRNA and the production of TGF-beta1 on macrophage-like cells which infiltrated in the dermis and the fibroblastic cells in BLM-induced scleroderma. Furthermore, HGF gene transfection prevented and ameliarated not only dermal sclerosis but also lung fibrosis induced by subcutaneous BLM injection. These results indicated that gene therapy by the transfection of the human HGF cDNA may thus be a useful therapy for ssc and lung fibrosis involved in ssc.

0615

Molecular and cellular analysis of a Japanese patient with xeroderma pigmentosum complementation group D who has the clinical features of Cockayne syndrome

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Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are distinct disorders associated with defects in excision repair of UV-induced DNA damage. In addition to the classical forms, some patients show characteristics of both diseases (XP/CS complex). Complementation studies have revealed that these patients belong to XP groups B, D and G, and only two XPD/CS patients have been reported. Here we report a new individual with the XPD/CS complex, who expired at the age of 2 with squamous cell carcinoma and liver failure. The level of NER measured as UDS was 33 % that of normal cells. As with the two previously described XPD/CS patients, the new patient's cells introduce uncontrolled breaks into their genomic DNA following UV-Irradiation. The mutations previously identified in two XPD/CS patients locate in the C-terminal third of XPD protein, which is involved in interactions with another subunit of the TFIIH complex, designated p44. This interaction stimulates the helicase activity of the XPD within the TFIIH complex. In contrast, we have identified one causative mutation in the XPD gene as a G to A transversion at base 217 in the allele inherited from patient's mother, which was confirmed by the PCR-RFLP method. This results in a gly47 to arg change in the 1st DNA helicase domain of the XPD protein. This mutation was detected previously in the heterozygous state in a XPD patient with severe symptoms (XP1NE). In our case, there is no parental consanguinity and the alleles are heterozygous. Standard bidirectional sequencing of cDNA did not reveal the mutation in the second allele. Using PCR on genomic DNA from the proband and his father, we were unable to detect any mutations in the 5'-UTR including the GC, CAAT and TATA boxes. Further study on this strain will give us important clues to unveil the mechanisms of DNA-repair abnormalities.

0616

Alterations of calcium homeostasis in Mal de Meleda

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Calcium has an established role in the homeostasis of mammalian skin, and a gradient of calcium concentration increasing from the basal layer to the stratum granulosum modulates keratinocyte proliferation and differentiation. Recent evidence revealed the importance of calcium pumps and nicotinic receptors in squamous differentiation. Mal de Meleda (MdM) is an autosomal recessive inflammatory palmoplantar keratoderma in which we have identified mutations in the gene encoding for SLURP-1 (Secreted Ly-6/uPAR-related protein 1). Analysis of SLURP-1 transcripts by Northern blot and RT-PCR revealed that SLURP-1 is expressed in palmoplantar skin and in foreskin. SLURP-1 has homologies with the Ly-6/uPAR/neurotoxin protein superfamily characterized by conserved disulfide bridges and a C-terminal consensus sequence CCXXXXCN. This family includes CD59 (protectin), E48 antigen and lynx1 which modulates nAChRs in the CNS. Contrary to other family members, SLURP-1 lacks a GPI anchor and contains a predicted signal peptide sequence, suggesting that it is a secreted protein. Indeed, 293T cells transfected with a mammalian SLURP-1 expression vector secrete the protein into the culture medium. Moreover, tagging at both N- and C-termini, allowed to demonstrate that the signal peptide of SLURP-1 is proteolytically processed during secretion. N-Glycosidase assays of transfected mammalian and insect cells, indicate that SLURP-1 is not glycosylated even tough this is predicted on N64 in silico. Finally, we tested whether SLURP-1 could directly modulate nicotinic receptors, similarly to lynx1. Recombinant 6XHis-tagged SLURP-1 was produced in mammalian 293T cells, purified with Ni-NTA resin, and used to assess the effect of SLURP-1 on human α7 nAChRs expressed in Xenopus oocytes. Indeed, it appears that SLURP-1 interacts with α7 nAChRs and is able to modulate the amplitude of the ACh-evoked currents. We hypothesize that SLURP1 modulates intracellular calcium content in keratinocytes, thus acting as a signal in normal growth and development of palmoplantar keratinocytes.

0617

Cooperation of connexin 30.3 and 31 is essential for assembly and stability of connexons

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Connexins are homologous proteins with 4 transmembrane domains, major components of gap junctions. We have recently identified mutations in either connexin 31 (Cx31) or 30.3 (Cx30.3) to be causally involved in erythrokeratodermia variabilis (EKV), an autosomal dominant disorder of keratinization. The F137L mutation in either Cx31 or Cx30.3 leads to EKV. This amino acid is highly conservated in connexin family and is oriented towards the channel pore. However the mechanism by which the Cx30.3 F137L mutation leads to the disease is unclear and may occur either by a dominant negative effect or by haploinsufficiency. Here we studied the effect of this mutation on expression, trafficking and localization of cotransfected Cx31 and Cx30.3 in connexin-deficient HeLa cells. Cx31 and Cx30.3 cDNAs were cloned into a selectable mammalian expression plasmid allowing the coexpression of both inserts with fused tags. Different plasmids with various combination of inserts (WT Cx31, WT Cx30.3 or M Cx30.3) have been constructed. HeLa cells transfected by these plasmids expressed high levels of RNA and proteins. Coexpressed WT Cx31 and WT Cx30.3 colocalized and appeared as large plaques at the plasma membrane as revealed by an anti N-Cadherin antibody indicating that Cx are addressed to the cell membrane. Moreover we were able to coprecipitate Cx30.3 and Cx31 under these conditions indicating that both connexins interact physically. WT Cx31 and Cx30.3 alone are located at plasma membrane, at cell-cell contacts but as small plaques or dots. Contrary, when mutant Cx30.3 is coexpressed with WT Cx31, the staining remains diffuse and mainly in the perinuclear space. In conclusion, our results indicate the importance of quality control mechanism in the assembly of Cx30.3-31 connexons before they reach the plasma membrane.

0618

Gene expression profile in keratinocytes from patients with recessive dystrophic epidermolysis bullosa following scratch wounding

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Generalized recessive dystrophic epidermolysis bullosa (RDEB) results from loss-of-function mutations in the anchoring fibril protein, type VII collagen. Clinically, there is trauma-induced blistering, chronic erosions and poor wound healing. The aim of this study was to examine gene expression profiles to assess the early molecular events in RDEB keratinocytes in response to injury. Primary confluent keratinocyte cultures were established from 3 RDEB subjects and 3 controls. RNA was harvested at baseline, at 6 hours and at 12 hours after scratch wounding. cDNA was hybridized to DermArray Human GeneFilters and the results were quantified using Pathways™ software. Only changes above two-fold were considered significant and the results were confirmed by semi-quantitative reverse transcription PCR. In RDEB wounded cells, three genes were downregulated at 6 hours compared with controls. These were psoriasin 1, stromelysin 2 and ubiquitin-conjugating enzyme E2G2 (UBE2G2). There were no significant changes at any other time-points. Psoriasin 1 is an S100 EF-hand calcium-binding protein with putative roles in calcium signal transduction, cell differentiation and cell cycle progression, although its precise function in keratinocyte cell biology is unknown. Stromelysin 2 is a metalloproteinase family member and is highly expressed in chronically inflamed diabetic and venous ulcers, but no previous data exists on its expression in either *in vivo* or *in vitro* RDEB wounds. UBE2G2 is an enzyme in the ubiquitin pathway that is relevant to cell homeostasis in several biological contexts. In summary, our study suggests that there are significant differences in the gene expression profile of RDEB keratinocytes compared to controls during the early phase of wound healing. These differences may have relevance to cell migration and keratinocyte proliferation and provide insight into the pathological mechanisms that contribute to delayed and poor wound healing in RDEB.

0619

Wound healing's transcriptional signature in epidermis of normal human skin

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As a complex process involving highly coordinated, interacting regulatory pathways in different cell types, wound healing is a prototype biological process to be studied by gene array technology. Using this technology we have the ability to advance beyond the analysis of individual genes, and analyze globally functional pathways and interactions of cellular components that regulate and carry out cellular processes. To determine the transcriptional patterns of the normal wound healing process in epidermis we performed gene array analysis of the wounded normal human skin at 4, 48 and 96 hours post-wounding using Affymetrix HU95 chips. We found 930 genes to be significantly regulated (14.7% of all genes found expressed). Overall, we found that the 4 and 48 hours transcriptional patterns are more similar to each other than to those at the 96 hours time point. As expected, we found that the adhesion molecules (such as desmosomal components) are initially suppressed (4 and 48 hours) whereas they start to increase at the 96 hours time point. Tight junction genes were strongly suppressed 4 and 48 hours post-wounding thus indicating junctional rearrangements that are expected from the cellular preparation for movement. At the 96 hours these genes became derepressed - their levels were restored to the levels prior to the wounding. In addition, MMP1, 2 and 10 were induced at 4 and 48 hours time points. Cell cycle and DNA synthesis genes, although expressed in early time points, are strongly induced at the 96 hours point. This is rather unexpected, because it was assumed that migration and proliferation overlap in time, and in combination is one of the initial keratinocyte's responses to wounding. Our results indicate that in response to wounding, keratinocytes first migrate and subsequently divide. This kinetics also correlates with the induction of steroidogenic enzymes. Taken together, our results show very specific regulatory pattern leading to particular keratinocyte phenotype during wound healing.

0620

Large-scale genomic approach to identify diagnostic/ prognostic marker and drug target candidate genes linked to skin diseases

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The aim of our studies is to identify drug target candidate genes and diagnostic/ prognostic marker genes linked to inflammatory skin diseases and skin cancers. In a pilot project, we constructed two SAGE libraries (30000 tags) using RNA of 21 psoriasis biopsies and of 22 healthy individuals respectively. Comparison of the two SAGE libraries revealed about 70 differentially (>2-fold, $p<0.01$) expressed genes, some of them already known to be associated with psoriasis, like protease inhibitor 3, keratin 6A or the S100 calcium binding proteins, e.g. psoriasin 1. Confirmation of our results was achieved using the PIQOR array technology. Each of the psoriasis biopsies and the healthy controls were hybridized versus a common reference on PIQOR Skin1.0 arrays. The PIQOR Skin1.0 array comprises about 1300 genes expressed in human skin diseases or normal skin. The expression profiles of individual patients allowed us to distinguish between genes differentially expressed in individuals or subgroups, and putative universal marker genes for psoriasis. Our results clearly demonstrate that SAGE data from pooled samples should always be confirmed by analysis of individual biopsies. Otherwise the identification of putative marker genes allowing a new pathological classification of the disease might be missed or individual differences in gene expression might be misinterpreted as universal.

0621

Mutations in a novel actin-extracellular matrix linkage gene cause Kindler syndrome

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Kindler syndrome (OMIM 173650) is a very rare, autosomal recessive genodermatosis with skin fragility and diffuse poikiloderma. A genome-wide scan on 26 patients from a genetically isolated Amerindian tribe in Panama mapped the disorder to 20p12.3. Haplotype analysis reduced the region to 0.6 cM between D20S905 and D20S194 containing 15 genes. This was confirmed in patients from the United States, Britain, Italy, Oman, Jordan, Turkey, Saudi Arabia, Afghanistan, Pakistan, and Japan by linkage and/or homozygosity mapping. Loss-of-function mutations were identified in the FLJ20116 gene (KIND1 encoding the protein kindlin) by direct sequencing of genomic DNA. The patients from Panama, the United States and Oman had nonsense mutation R271X. Nonsense mutation R288X was detected in both a British and a Turkish patient and Q65X was detected in a Jordanian patient. A splice site mutation in was found in Saudi Arabian patients. In all cases, the affected were homozygous for the null-allele. Kindlin is the human homologue of the C. elegans protein UNC112, a membrane-associated structural/signaling protein that has been implicated in linking the actin cytoskeleton to the extracellular matrix. Immunofluorescence labeling with polyclonal rabbit anti-kindlin antibody showed markedly reduced staining in KS patient epidermis, particularly in keratinocytes, compared with controls. Prominent reduplication of the lamina densa was seen on electron microscopy, consistent with the proposed function of kindlin by analogy to UNC112. This is the first demonstration of a defect in actin-extracellular matrix linkage, rather than keratin-extracellular matrix linkage, causing an inherited skin fragility syndrome.

0622

Genetics of proliferative activity of keratinocyte stem cells *in vitro*

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Several converting lines of evidence suggest that the cellular targets for carcinogenesis and tumor promotions in the cutaneous epithelium are keratinocyte stem cells, including in vitro clonogenic cells with high proliferative potential. These observations indicate that understanding the regulation of keratinocyte stem cell number and proliferative potential is critical for understanding the development of skin cancer. To address this problem and to look toword the identification of stem cell regulatory genes, we have tested two inbred mouse strains, C57BL/6 and BALB/c and their hybrid (BALB/cxC57BL/6)F1 in a sensitive and quantitative in vitro assay for clonogenic keratinocytes (KCs). Following examination of 4000 KCs, we found that the average size, the area of all colonies/dish, and the area of the largest colony/dish were always higher in the C57BL/6 then in the BALB/c mice. Their (BALB/cxC57BL/6)F1 hybrid had a KC size intermediate between the parental strains. Hence, the average KC size in C57BL/6 mice was 2.3 mm2, in BALB/c - 1 mm2, while (BALB/cxC57BL/6)F1 was 1.6 mm2. The average area of colonies/dish in C57BL/6 mice was 191.9 mm2, in BALB/c - 52.8 mm2, while (BALB/cxC57BL/6)F1 was 102.3 mm2. The maximal area of an individual colony was 12.6 mm2 C57BL/6, 4.9 mm2 in BALB/c, while 9.9 mm2 in (BALB/cxC57BL/6)F1 mice. We conclude that KC size is probably genetically regulated. This finding sets the stage for the mapping and identification of genes involved in the proliferative activity of keratinocyte stem cells. This research was supported in part by NIH grants CA45293, CA87780, and CA97957 and by grants from the Lankenau Foundation (RJM).

0623

Retroviral-mediated gene transfer of latency associated peptide into murine bone marrow cells and generation of LAP-producing macrophages

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Monocyte/macrophage cells play a critical role in fibrosis via TGFβ1. Latency associated peptide (LAP) is a natural antagonist to TGFβ1 that binds to and inactivates mature TGFβ1. We have shown that recombinant human LAP (hLAP), when administered intravenously, can inhibit fibrosis in mice with sclerodermatous graft versus host disease (Scl GVHD), a model for human scleroderma. To establish a gene therapy for scleroderma and Scl GVHD through expression of hLAP in monocyte/macrophages, a bicistronic murine stem cell virus (MSCV) retroviral vector encoding hLAP, an internal ribosome entry site (IRES), and the green fluorescent protein (GFP) for detection was used to overexpress the hLAP protein. Stable retroviral producer cell lines were established by transfecting packaging cell line GP+E86 with the hLAP MSCV retroviral vector. B10.D2 mouse bone marrow (BM) cells were then successfully transduced by the stable retroviral producer cells, and driven *in vitro* into monocyte/macrophages using GM-CSF. We characterized the transduced BM cells by flow cytometry for stem cells, macrophages and dendritic cells (CD34, CD11b, CD11c and DEC205). We also showed expression of hLAP by ELISA and western blot analysis of supernatants. Our data demonstrate that most of the transduced cells after 10 days in culture are $CD11b^{+}$, and the overexpressed hLAP does not affect their differentiation into macrophages.

0624

Identification of a locus for type I punctate palmoplantar keratoderma on chromosome 15q22-23

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The identification of the molecular basis of disorders of keratinization has significantly advanced our understanding of skin biology, revealing new information on key structures in the skin such as the intermediate filaments, desmosomes and gap junctions. Among these disorders, there is an extraordinarily heterogeneous group known as palmoplantar keratodermas, for which only a few molecular defects have been described. Here, we report the identification of the first locus for type I punctate palmoplantar keratoderma (MIM# 148600). A genomewide scan was performed on an extended autosomal dominant pedigree from Israel and linkage to chromosome 15q22-23 was identified. With the addition of two new families with the same phenotype, from Mexico and Israel, we confirmed the mapping of the locus for punctate PPK to a 9.98-cM interval, flanked by markers D15S534 and D15S818 (maximum two-point LOD score of 4.23 at q=0.01 for marker D15S988). No obvious candidate genes were found in the critical linkage interval. These findings will facilitate the identification of a new gene involved in skin integrity that could also contribute to the understanding of the multiple organ involvement in the syndromic forms of PPK, associated with different types of malignancies, hearing loss and cardiomyopathy, among others.

0625

Expression of DDR2 and metalloproteinases in human melanocytes and melanoma cell lines

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Matrix metallopoteinases (MMPs) degrade the extracellular matrix and facilitate tumor invasion and metastasis. There is strong evidence that DDR2 (a tyrosine kinase localized to chromosome lq21-23) is capable of up-regulating certain MMPs. If the expression of DDR2 correlates with the expression or activation of MMPs in melanoma, this could identify DDR2 as a novel target for MMP regulation. Therefore, we used microarrays, differential tyrosine kinase display, real-time PCR, fluorescent in-situ hybridization (FISH) and Northern blot analysis to study the expression of DDR2, MMP1 and MMP2 in normal human epidermal melanocytes and three human melanoma cell lines:Hs-294T, G-361 and A-375. Evaluation of gene expression indicated that DDR2, MMP1 and MMP2 are overexpressed in melanoma cell lines relative to normal melanocytes. To determine if DDR2 overexpression was due to gene amplification,we used FISH to determine DDR2 copy number and correlated it with DDR2 expression. Expression of DDR2 did not parallel the DDR2 gene copy number. The highest DDR2 expression was observed in the Hs-294T line with only 3 copies of the DDR2 gene. The second highest expression was observed in the G-361 line, which had up to 6 copies of DDR2 due to the presence of chromosomal rearrangements containing the DDR2 gene,although instability of this line led to most cells losing one or another of the chromosomes containing DDR2. The A-375 cell line had the lowest gene expression, despite having three or more copies of the chromosome 1 segment containing DDR2. Therefore, the level of DDR2 gene expression does not appear to be directly related to its gene copy number, but rather may be controlled by genes at other loci that promote or are required for DDR2 expression.This may prove relevant to understanding the role of DDR2 in regulating the expression of MMPs in human melanomas.

0626

Clinical and pathological correlations in genetically-distinct forms of atrichia

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The genetic basis of two distinct forms of atrichia with papules has recently been defined at the molecular level. In atrichia with papular lesions (APL, OMIM 209500), a rare autosomal recessive disease characterized by early-onset of atrichia, followed by a papular eruption, mutations in the hairless gene (HR) on chromosome 8p21 have recently been identified. Atrichia with papules also occurs in the clinical setting of vitamin D-dependent rickets type IIA, also known as Rickets-Alopecia syndrome, (VDRRIIA, OMIM 277440), resulting from mutations in the vitamin D receptor gene (VDR) on chromosome 12q12-q14. Despite the distinct genetic basis for both forms of atrichia, the clinical findings are strikingly similar and exhibit classical pathognomonic features unique to this phenotype. In this study, we sought to document the clinical and molecular features of APL and VDRRIIA in three families with APL and two families with VDRRIIA. Histological analysis and detailed clinical histories were taken from affected individuals, and molecular analysis of the HR and VDR genes was performed on genomic DNA from probands and family members. We underscore the genetically heterogeneous basis of atrichia by identification of several novel pathogenetic mutations in the HR and VDR genes in all families studied. Increased awareness of these two diseases will allow early diagnosis and potential therapeutic intervention for the rickets in VDRRIIA, and avoidance of treatment of the atrichia in both APL and VDRRIIA.

0627

Injection of genetically-engineered fibroblasts corrects regenerated human epidermolysis bullosa skin tissue

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Current therapeutic strategies for genodermatoses rely on grafting genetically engineered epidermal tissue. Drawbacks limit applying this approach to humans, including the fragility of engineered epidermal tissue and the need to remove existing tissue prior to grafting. Because fibroblasts are robust cells that synthesize and secrete extracellular matrix, we explored their utility in recessive dystrophic epidermolysis bullosa (RDEB), a disorder due to defective extracellular type VII collagen. Primary fibroblasts from 4 previously characterized RDEB patients with COL7A1 mutations were engineered for >4-fold human type VII collagen overexpression. Intradermal injection of these cells into intact RDEB skin regenerated on scid mice stably restored correctly localized type VII collagen expression in vivo and normalized hallmark RDEB disease features, including sub-epidermal blistering and anchoring fibril defects. Expression was stable for the 16-week duration of the experiment. BMZ-localized collagen VII was detected up to 75um away from the nearest collagen VII[+] cells in the dermis, as verified by serial 5um cryosections, indicating that the fibroblast-produced protein can be delivered from sites many cell diameters away in the tissue. Of interest, regions more distant from the intradermal injection site displayed blistering in areas transitioning to absent collagen VII protein, demonstrating the corrective impact of restoring this protein on tissue cohesion. Collagen VII overexpression at higher levels than normal is required in this approach because normal fibroblasts failed to deliver detectable protein to the overlying epidermal basement membrane zone. Injection of genetically engineered fibroblasts thus provides a simplified approach to correcting human disorders of secreted matrix proteins.

0628

Does the position of premature termination codon in COL7A1 correlate to the severity of the clinical phenotype in recessive dystrophic epidermolysis bullosa?

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin disease in which type VII collagen is a responsible protein. It has been reported that the position of premature termination codon (PTC) in the type VII collagen gene (COL7A1) correlates with the clinical severity in RDEB by presenting three cases possessing different combination of recurrent mutations, 5818delC (exon 70), 6573+1GtoC (intron 81) and E2857X (exon 116). However, we have experienced a notable exceptional case of mitis RDEB that possessed upstream PTC combination but showed very mild phenotype. The patient was a 9-month-old Japanese male with no family history of blistering skin disorder. Erosions and blisters had been present on acral skin at birth, but generalized blistering or fusion of digits has not been observed until the age of two. The patient and the father had a single nucleotide deletion within exon 64 (5504delA), which has not been previously reported, and causes a frame shift and exchange of five amino acids before creating a PTC. The adenine deletion also creates GT sequence and is supposed to have potential to be recognized as splicing donor site that splices out 30 bps including the PTC. However, reverse transcriptase (RT)-PCR showed the single base deletion causing the frame shift and PTC, and alternative products were not detected. The patient and the mother were shown to have a recurrent donor splice site mutation at intron 81 (6573+1GtoC), and confirmed by RT-PCR to cause activation of cryptic donor splicing site resulted in downstream PTC as reported previously. Although the PTCs in our case located more upstream, clinical phenotype was apparently milder than three reported cases. There is still a possibility that a small amount of mRNA undetectable by RT-PCR produces truncate protein. Otherwise the additional factor that affects ability to form anchoring fibrils should be considered for the elucidation of the genotype-phenotype relationship in RDEB.

0629

A novel clinical subtype of epidermolysis bullosa simplex associated with pyloric atresia is caused by plectin gene (*PLEC1*) mutations

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Epidermolysis bullosa (EB) can be divided into three major subtypes, EB simplex (EBS), junctional EB (JEB), and dystrophic EB, according to the level of basement membrane zone (BMZ) tissue separation within the basal keratinocyte, lamina lucida, sub-lamina densa, respectively. While *PLEC1* mutations cause EBS associated with muscular dystrophy (EBS-MD), JEB associated with pyloric atresia (JEB-PA) results from mutations in the genes (*ITGA6* and *ITGB4*) encoding the hemidesmosome integrin α6β4. In this study, we identified three individuals from two families with EBS associated with pyloric atresia (EBS-PA) caused by *PLEC1* mutations. Electron microscopy of the patient's skin revealed an intraepidermal plane of cleavage, hypoplastic hemidesmosomes (inner plaques) and a reduced keratin filament association. While immunofluorescence showed normal expression of α6β4 integrin, monoclonal antibodies against plectin, a large multifunctional plakin protein, were completely absent or markedly attenuated in the BMZ of the two patients. Sequence analysis of genomic DNA demonstrated that both probands harbored novel mutations in *PLEC1*. One patient was compound heterozygous for a premature termination codon (PTC) and a splice-site mutation (Q305X/1395G->A). The other patient harbored homozygous PTCs (R1189X) as well as a heterozygous PTC (Q2538X); the former was of paternal origin and the latter was of maternal origin. Intragenic polymorphism analysis suggested that the occurrence of the homozygous PTC (R1189X) was due to segmental uniparental disomy. It is well known that *PLEC1* mutations can lead to EBS-MD, but there have been no reports in which *PLEC1* mutations lead to EBS-PA. These results confirm the existence of a novel subtype of EBS-PA resulting from *PLEC1* mutations, and provide further information about the roles of plectin/α6β4 integrin in intestinal development and in the classification of EB.

0630

Identification of factors which interact with the calcium response element of human keratin 1 and analysis of its functional role

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We have previously identified a functional calcium responsive element (CaRE), 3' of the human K1 gene that consists of a composite AP-1/steroid hormone element. In the present study we demonstrate that in a low calcium environment, when the CaRE is unable to activate a target promoter, the AP-1 site is occupied by either c-Jun or JunB and the hormone response element is bound by COUP. Overexpression of c-Jun, or COUP in primary keratinocyte cultures cotransfected with a CaRE reporter construct specifically suppressed calcium activation of the promoter by the CaRE. In contrast, we found that overexpression of c-Fos in primary keratinocytes activated the CaRE reporter construct in the absence of calcium while c-Jun, JunB or JunD did not. These results suggest that Jun and COUP bind to the CaRE in undifferentiated keratinocytes to suppress transcription of HK1. Upon initiation of differentiation, c-Fos displaces Jun from the CaRE to activate expression of K1 in post-mitotic keratinocytes. Glutathione S-transferase pull down assay demonstrated the direct binding of COUP to c-Jun and c-Fos in vitro. The binding affinity of COUP to c-Jun seemed to be higher than that to c-Fos, suggesting that COUP and c-Jun can cooperatively suppress the expression of HK1 in undifferentiated keratinocytes via their direct interaction. Taken together, these data provide evidence for both physical and functional interactions among COUP and AP-1 factors during differentiation of keratinocytes.

0631

A novel member of the S100 family is alternatively spliced and overexpressed in psoriatic skin

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By gene expression profiling of psoriatic skin, we have identified a new member of the human S100 family. Differential display screening of normal and psoriatic skin revealed a cDNA (clone 110) that is highly upregulated in lesional skin. Initial sequence analysis showed high homology to a non-coding cDNA. By subsequent cDNA cloning of psoriatic skin, a corresponding coding region which is present in two alternatively spliced mRNA-isoforms was identified. The splice variants detected are about 0.5 kb and 4.4 kb in length and differ in their untranslated regions. Both isoforms share a coding region predicting a protein of 101 amino acids that shares high homology to the S100A7 protein. Analysis of the deduced amino acid sequence revealed two potential EF-hand motifs as Ca^{2+}-binding domains. By sequence comparison, we have localized this gene on human chromosome 1q21, within the region of the epidermal differentiation complex where other members of the S100 family are clustered. Analysis of genomic DNA revealed that the gene is split into three exons and two introns spanning a total of about 7 kb. Increased expression of the mRNA-splice variants in psoriatic skin was confirmed by RT-PCR and Northern blot analysis. *In-situ* hybridization revealed a markedly increased number of mRNA molecules in the basal and suprabasal epidermal layers of psoriatic skin when compared to healthy skin tissue. Our data on expression and distribution of this gene suggest that it may be involved in processes of epidermal proliferation and differentiation and might be of pathophysiological importance in psoriasis.

0632

Photoprotective gene transfer in keratinocytes

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We have previously demonstrated that UV irradiation induces direct and oxidative DNA damage, protein and lipid oxidation and subsequently sunburn cell formation in epidermal reconstructs. The UV-induced generation of free radicals is associated with a decrease in the activity of the two major antioxidant enzymes, catalase and superoxide dismutase (SOD). Catalase and SOD activities are down-regulated respectively by superoxide anion and hydrogen peroxide and their oxidation at the protein level diminish their activities. Since SOD induces the formation of hydrogen peroxide, the substrate of catalase, a change in the SOD/catalase ratio may be more deleterious than an equal decrease of the two activities. We have previously shown that the SOD/catalase ratio is higher in low phototype skin and increases with the content in linoleic acid, the most UV-oxidation prone polyunsaturated fatty acid. We have demonstrated further that sunscreens and antioxidant molecules protect efficiently against sunburn cell formation without protecting completely DNA, leading to mutations, which might cause skin cancer in fair skinned individuals. Thus, we have hypothesized that a "reinforcement" of enzymatic antioxidant defences may help protecting against skin cancer in high risk individuals. To evaluate this strategy, we have constructed bicistronic lentiviral vectors with catalase (and EGFP as marker) or SOD and (DsRed2 as marker). Our preliminary research shows that 293Tcells transfected by catalase/EGFP or SOD/ DsRed2 have increased enzymatic activity in correlation with an increased protein expression and an increased number of fluorescent cells. The UV-induced decrease in catalase activity was less important in keratinocytes transduced by catalase/EGFP than in non-transduced keratinocytes. The transgene was correctly expressed and stable in epidermal reconstructs. The long term effect of the transgene will be studied using xenografted reconstructs in immunodeficient mice.

0633

Transduction of monocyte-derived dendritic cells with lentiviral vectors for melanoma immunotherapy

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Genetically engineered dendritic cells (DCs) presenting specific antigens (Ags) to T cells may be of great interest for cancer immunotherapy. The expression of GFP under the control of different promoters (EF-1alpha, CMV or PGK promoters) was measured by transducing either human monocytes or immature/mature DCs with lentivectors (LVs). Up to 70% of human mature DCs expressed GFP. Human immature DCs could be further differentiated with LPS into mature DCs expressing high levels of costimulatory molecules, showing that LV transduction did not affect the normal maturation process. In addition, transduction efficiency was evaluated in human Good Manufacturing Practice (GMP) mature DCs with serum-free conditions. Over 90% GFP expression was obtained with no significant apoptotic effect. Furthermore, we developed novel tools to target transgene expression using LVs containing a CD83 DC specific promoter. The specificity of these vectors targeting DCs is demonstrated on cell lineages generated from transduced CD34+ hematopoietic stem cells as well as on monocyte-derived DCs. Comparison with LVs bearing HLA-DRalpha or MHC class II transactivator promoters will be shown. In addition, results showing transduction of human DCs with LVs encoding for siRNA specific for surface receptors important for DC function will be presented. We are currently investigating in vitro specific immune responses elicited by human DCs transduced with lentivectors encoding for melanoma-associated Ags. Altogether these results point to lentivectors as promising candidates for DC-based immunotherapy purposes due to high transduction ability, targeting properties and capacity of inducing Ag-specific immune responses.

0634

Refinement of PSORS1 by haplotype sharing analysis: non-conserved mutations in an intact retroviral dUTPase open reading frame

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The nature of the PSORS1 mutation is still enigmatic. We previously developed a haplotype sharing test as a tool to refine association mapping. Using this method we narrowed the target region to a 7 kb interval around marker M6S168 by re - analyzing published data (Nair et al, Am J Hum Gen, 2000). This result was highly significant (increase of - log p value from 4,5 at nearby markers to 11,9 at M6S168). This finding was confirmed in an independent patient sample containing 423 patient and 226 control haplotypes. To identify mutations distinguishing the disease - associated haplotype, we pooled DNA from patients homozygous for a high risk haplotype (n = 10) and DNA from control probands homozygous for a non-disease associated haplotype (n = 4) differing from the high-risk haplotype only at marker M6S168. Both DNA pools were sequenced over a length of 4.2 kb telomeric of M6S168, focussing on a region containing endogenous retroviral sequences. A total of 17 base pair variations between both pools were identified, yielding an average of one bp exchange per 250 base pairs. Four of the identified base pair exchanges were clustered in an open reading frame encoding an HERV-K derived dUTPase containing all five conserved protein motifs. Two of these mutations encode predicted amino acid exchanges (R119 ->G; K122 ->R) between the fourth and the fifth conserved enzyme motif. Our data confirm and refine the PSORS1 locus previously mapped by Nair et al. to a 10 kb interval and suggest that PSORS1 may not reside in a classical gene but rather exerts its effect through modification of endogenous retroviral sequence activity.

0635

Haplotype analysis of distantly-related populations implicates corneodesmosin in psoriasis susceptibility

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Psoriasis is a common skin disorder, inherited as a multifactorial trait. A major locus underlying disease susceptibility has been mapped to chromosome 6p21, within a 150 kb segment of the Major Histocompatibility Complex class I region. This interval contains 3 genes carrying psoriasis associated alleles: HLA-C, alpha-Helix Coiled coil Rod homologue (HCR) and Corneodesmosin (CDSN). We have recently derived ancestral haplotypes spanning these genes and identified a rare chromosome (cluster D), originating from a double recombination event that had replaced the region containing HCR risk alleles, while preserving the genomic segments that encompass the HLA-C and CDSN disease-associated SNPs. This observation suggested that CDSN SNPs might be required to confer psoriasis susceptibility, together with HLA-C risk alleles. In the present study we have analysed 6 coding and 10 non-coding CDSN SNPs in 171 UK and 30 Indian parent-offspring trios. We have identified a 16 SNP chromosome showing significant disease association in both datasets ($p < 10^{-10}$ and $p = 3.9 \times 10^{-3}$, respectively) and occurring on both sets of haplotypes (clusters D and E) known to confer psoriasis susceptibility. Altogether, our results support a disease model requiring the presence of CDSN susceptibility alleles on haplotypes bearing HLA-C related risk SNPs, as the data provide evidence that these risk alleles have not been maintained together solely by linkage disequilibrium.

0636

Topically-regulated control of hematopoiesis via direct cutaneous gene transfer

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The capacity for direct and topically controllable gene delivery to intact human skin tissue represents a major goal in cutaneous gene transfer. Among gene transfer vectors, lentiviruses are attractive for this purpose because they infect both dividing and non-dividing cells within skin after direct administration. HIV-1-based lentiviral vectors were generated in which gene expression is driven by 5 high-affinity glucocorticoid responsive elements placed upstream of the adenovirus 2 major late promoter core TATA sequence. As a product for regulated gene delivery, we used erythropoietin (EPO) because of the need to control the stimulation of hematopoiesis. Furthermore, we had previously demonstrated that a single intradermal injection of an unregulated CMV-driven EPO lentivector to human skin grafted on scid mice effectively delivers EPO protein to the bloodstream and boosts hematocrit dramatically. 5ug of p24 equivalent of the regulated EPO lentivectors were injected into full thickness human skin grafts on scid mice (n=5/group). 7 days later, glucocorticoid was administered, as either three consecutive administrations of 60ug intraperitoneal dexamethasone or as topical clobetasol propionate. Serum levels of human EPO rose from undetectable to a peak of 23mIU/ml in 3 days. 2 cycles of glucocorticoid stimulation led to corresponding rises and falls in hematocrit from an average of 42% at baseline to a peak of 67.5% 10 days after stimulation. Topical stimulation with clobetasol was as effective as systemically administered dexamethasone. Regulated lentivectors thus provide a platform for regulated and reversible direct cutaneous gene delivery of therapeutic polypeptides to the systemic circulation.

0637

Gene transfer to cells and skin tissue via reversible plasmid condensation with protein transduction domains

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Nonviral gene transfer offers biosafety, stability and expense advantages over viruses, however, it has suffered from poor efficiency. Recently, new molecular transporters in the form of protein transduction sequences (PTS) derived from proteins such as HIV1 TAT and Drosophila antennapedia have been found to rapidly traverse cell and visceral tissue barriers, including the blood-brain barrier. Carrying cargo up to 40nm in size, these transporters appear to rely on arginine residues that are also present in evolutionarily conserved sperm protamines that facilitate delivery of macromolecules such as the male haploid genome. Since arginine-rich peptides facilitate uptake of macromolecules such as proteins, liposomes and iron nanoparticles, we explored their potential in enhancing plasmid DNA delivery. In their unmodified form, known protein transduction sequences, including hepta-arginine and Tat47-57, failed to support effective gene delivery. However, by flanking a core of consecutive arginines with amino-, and carboxy-terminal cysteines in vitro gene transfer was observed. Furthermore, interspersing arginines with glycine and histidine residues achieved reversible plasmid condensation and dramatically increased transfection levels in a variety of cell types, including primary human fibroblasts. Gene transfer efficiency was superior in most settings to benchmark commercially available approaches. Unlike most available cationic homopolymers that function only in vitro, these new peptides also increased gene expression in both murine and human tissue in vivo, leading to >4.5 fold increased levels over naked DNA as measured quantitatively by in vivo bioluminescence. Thus, cysteine-flanked, internally spaced arginine-rich (CFIS-R) peptides represent a new approach to efficient nonviral plasmid delivery using rationally designed protein transduction domains.

0638

Phenotype-genotype corelation of a xeroderma pigmentosum variant patient

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We examined the clinical, molecular, and genetic features of a newly diagnosed xeroderma pigmentosum variant (XPV) patient. XP3GO is a 33 y old Caucasian male who is not sun sensitive. Around puberty he noticed freckling in the UV-exposed areas of his skin. At age 22 he started to develop skin cancers including multiple primary melanomas, but also basal cell and squamous cell carcinomas. There is no known consanguinity of his parents. We established a XP3GO fibroblast strain and found normal DNA repair levels as reflected by normal host cell reactivation of a UV-treated luciferase gene-containing reporter plasmid. Post-UVC cell survival of XP3GO fibroblasts was normal, but was reduced in the presence of 2 mM caffeine, a hallmark of XPV cells. XPV cells are defective in polymerase eta. Western blot analysis revealed undetectable levels of polymerase eta protein. Mutational analysis of the polymerase eta gene showed a deletion of exon 6 in cDNA due to a homozygous deletion of an about 1.3 kb region in the genomic DNA that includes exon 6. This deletion leads to a severely truncated and potentially non-functional polymerase eta protein. The ensuing loss of effective translesional synthesis past UV photoproducts produces an increased tendency of XP3GO cells to incorporate incorrect bases in DNA that results in a skin cancer-prone phenotype.

0639

Processing and staining of mouse skin for laser capture microdissection and nucleic acid retrieval

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In the study of disease, tissue heterogeneity limits the information available from the analysis of biological samples. To study genetic changes that occur in a particular cell type, tiny subpopulations of cells need to be isolated to reduce the risk of contamination. Laser capture microdissection (LCM) has been emerging as one of the methods of choice. The skin is an attractive organ for the application of LCM since it is composed of a complex mixture of different cell types. Here we describe the use of LCM in the analysis of an animal model of a hereditary blistering disorder, epidermolytic hyperkeratosis (EHK). We demonstrate that tiny subpopulations of keratinocytes can be readily isolated by LCM. Since our mouse model allows the induction of the phenotype in a circumscribed area of the skin, two genetically different populations of keratinocytes could be isolated from the same mouse. Microdissected keratinocytes from persisting hyperkeratotic lesions were a source of genomic DNA and were further subjected to PCR analysis. Cells from phenotypically normal skin served as a control. We demonstrate that LCM has proven a valuable and reliable tool in the isolation of pure samples of keratinocytes from different areas of mouse skin. The information obtained by LCM provided new insight into the molecular and cellular basis of mosaic skin disorders.

0640

A novel nonsense mutation of transglutaminase 1 gene in a pedigree with lamellar ichthyosis

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Autosomal recessive lamellar ichthyosis is a severe skin disorder characterized by generalized hyperkeratosis. Gene mutations of transglutaminase 1 (TGM1), which mediates cross-links in the formation of the cell envelop during epidermal terminal differentiation, have been identified as causes of lamellar ichthyosis. Previous genetic analysis has also demonstrated genetic heterogeneity in lamellar ichthyosis. Here we report that a novel homozygous nonsense gene mutation of TGM1, Q202X, in a Chinese patient with lamellar ichthyosis. Accordingly, his parents and one brother carry a heterozygous mutation. In addition, TGM1 transcript was undetectable in patient's skin by using RT-PCR and in situ hybridization. Further immunohistochemical study revealed the absence of transglutaminase 1 enzyme activity in patient's skin. Our findings suggest that this nonsense TGM1 mutation, Q202X, is responsible for the lack of mRNA transcripts and protein expression in the skin. As a consequence, this causes defective cell envelops in cornified layers, which in turn perturbs barrier function of the epidermis and leads to lamellar ichthyosis clinically.

0641

Transcriptional control of the Ese-2 gene by a novel keratinocyte-specific factor

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The epidermal differentiation process is controlled in part by transcription factors that regulate the expression of a myriad of genes that are unique to each stage of differentiation. We are studying Ese-2, an Epithelium-specific Ets domain transcription factor that is expressed only during the late stages of epidermal differentiation. Our goal is to identify and characterize the upstream signals and transcription factors that control the differentiation-specific expression of Ese-2. We have shown that the minimal promoter region of Ese-2 is in an open chromatin configuration as judged by the presence of a keratinocyte-specific DNAse I hypersensitive site. Serial deletion analysis has revealed that a 125 bp fragment of the Ese-2 promoter harbors most of the transcriptional activity as measured by reporter assays in transient transfection of keratinocytes. Interestingly, the Ese-2 promoter segment shows remarkable up regulation of transcriptional activity when the transfected keratinocytes are induced to differentiate by increasing the Ca++ concentration in the culture medium. We have performed a detailed study of this promoter segment by mutational analysis as well as by gel shift assays. Our studies have revealed a novel Keratinocyte-Specific Factor (KSF) that binds to the promoter of Ese-2 and confers most of the activity of the promoter. We are currently performing additional studies to determine the precise cis-elements that confer the differentiation-specific expression of Ese-2 promoter. Collectively these studies should provide new insights into the molecular mechanisms of epidermal differentiation.

0642

Cardiovascular disease in pseudoxanthoma elasticum is not linked to mutations in ABCC6

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Pseudoxanthoma elasticum (PXE) is a heritable multi-system disorder that affects the skin, eyes and cardiovascular system with vast intra and interfamilial variability. While clinical manifestations of the latter do not differ from those encountered in the aging population, in PXE they tend to occur at a much younger age and are associated with significant morbidity. We investigated the prevalence of cardiovascular disease among 60 families with PXE and attempted correlation to specific mutations in the ABCC6 gene, shown to underlie PXE. The most frequently encountered manifestations include claudication (19%) and coronary artery disease (12%), followed by GI bleeding and hypertension. These symptoms were not linked to one specific mutation or a particular protein domain. In addition, two large families with at least 3 siblings affected with PXE had prominent cardiovascular disease in affected and unaffected individuals. These individuals did not show segregation of a specific cardiovascular phenotype with their genotype. In fact, several siblings with PXE had no evidence of cardiovascular disease despite cardiological examination at an advanced age. Furthermore, we examined the prevalence of the common polymorphism R1268Q, which had previously been reported to be associated with hyperlipidemia. The frequency was equal in individuals with PXE and the general population and we could not confirm an association with this polymorphism and an altered lipid profile. These data suggest that there is no direct association between mutations in ABCC6 and cardiovascular disease, but it seems that early onset cardiovascular disease might be facilitated by an alternative pathomechanism in PXE.

0643

Sustained *in vivo* expression of human type VII collagen in mice by gene-corrected dystrophic epidermolysis bullosa fibroblast-mediated gene transfer

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Dystrophic epidermolysis bullosa (DEB) is a family of inherited mechano-bullous disorders caused by mutations in the type VII collagen (COL VII) gene and subsequent perturbations in anchoring fibrils. We previously used a lentiviral vector to express full-length COL VII in RDEB keratinocytes (KCs) and fibroblasts (FBs) and demonstrated the correction of the RDEB cellular phenotype *in vitro*. In this study, we evaluated the feasibility of using gene corrected RDEB FBs for gene therapy in an animal model. To accomplish this, 10 million gene corrected or uncorrected RDEB FBs or normal human FBs were injected intradermally into the skin of athymic hairless mice. Skin biopsies were obtained every week after injection and subjected to immunostaining using antibody specific for human COL VII . Gene-corrected RDEB FBs or normal human FBs were able to synthesize and deposit human COL VII within the mouse basement membrane zone (BMZ). The expression of human COL VII was sustained for at least 3 months (the last time point examined at this time), as were anchoring fibrils. Further, injected gene-corrected human RDEB FBs or normal human FBs were stably maintained in the mouse dermis. In contrast, mouse skin injected with parental RDEB FBs entirely lacked human COL VII staining at the BMZ. Interestingly, skin injected with gene-corrected RDEB KCs or normal human KCs intradermally failed to synthesize and deposit human COL VII within the mouse BMZ, and injected human keratinocytes were lost two weeks after injection. These results indicate that *in vivo* long-term, stable production of human COL VII can be achieved by direct application of fibroblast-mediated gene transfer. Since skin FBs can be passaged 30-60 times *in vitro* and are easy to grow in large numbers, it is likely that RDEB patients could be helped by gene correcting only their FBs *ex vivo*, culturing them in quantity and then injecting them back into the skin high in the dermis like injecting Zyderm for wrinkles.

0644

Rapid RFLP detection of mutations and functional alterations in the human XPD DNA repair gene

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Xeroderma pigmentosum (XP) is an autosomal recessive disease that is characterized by hypersensitivity to sunlight and markedly increased susceptibility to skin cancer in association with defective DNA repair. The XP gene, XPD, is a helicase that plays an important role not only in nucleotide excision repair but also in transcription as a member of the basal transcription factor complex, TFIIH. Defects in the XPD gene result in several different clinical phenotypes including XP with skin disease, XP with skin and neurological disease, the XP/Cockayne syndrome complex, or trichothiodystrophy (TTD), a disorder with brittle hair without increased cancer susceptibility. We analyzed cells from patients that have reduced post-UV cell survival and used a plasmid host cell reactivation (HCR) assay to assign 18 cell lines to XP complementation group D. We developed RFLP assays to detect 24 XPD missense mutations that have been reported. Eleven of the XP-D cell lines we tested had a common base substitution mutation, C2125T, which resulted in a missense alteration (R683W) in helicase domain VI. One of these patients was homozygous for this mutation. Five of these patients had XP with neurological disease, 2 had XP with only skin disease and 4 have unknown symptoms. We created an expression vector containing XPD cDNA with the C2125T mutation and found complete absence of function in the HCR assay. Three other patients had different mutations that were detectible by the RFLP assays. XPD mutations were not detected in 4 XP-D cell lines by these RFLP assays. RFLP assays may be used to rapidly identify mutations in XP cells in complementation group D.

0645

Topically regulated transgene expression in epidermal keratinocytes

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A major challenge for current gene therapy is controlling the expression level of therapeutic genes. An inducible gene-switch system was recently developed that allowed regulated expression of transgenes. The purpose of this study is to explore the possibility of using the epidermis as a secretory machinery to produce therapeutic proteins with the gene-switch system to treat systemic deficiencies. Epidermal keratinocytes have been shown to secrete proteins into the circulation via the vascular system within the dermis. We established a transgenic mouse model in which expression of a human growth hormone (hGH) reporter gene could be activated by topical application of an inducer. In the absence of an inducer, the Gene-switch activator protein was inactive, and hGH was not transcribed. In the presence of an inducer, the activator protein up-regulates hGH transcription in basal keratinocytes. High levels of hGH were detected in the circulation within hours after a single induction, with the maximum levels about 200-fold higher than controls. The serum levels of hGH were dependent on the amount of inducer applied, and repeated induction resulted in increased weight gain in transgenic versus control mice. Furthermore, physiological levels of hGH were detected in the serum of nude mice after topical induction of small transgenic skin grafts. These results clearly demonstrate the feasibility of using the gene-switch system to regulate the delivery of therapeutic proteins into the circulation via genetically modified keratinocytes. We are currently testing whether a similar transgenic system could be used for the treatment of hemophilia B in a mouse model.

0646

Development of *in vivo* gene therapy for dystrophic epidermolysis bullosa using newly engineered minimal lentiviral vectors

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Dystrophic epidermolysis bullosa (DEB) is a family of inherited mechano-bullous disorders caused by mutations in the type VII collagen (COL VII) gene and subsequent perturbations in anchoring fibrils. The lack of therapy for DEB provides an impetus to develop gene therapy strategies. An *ex vivo* approach for cutaneous gene transfer requires surgical transplantation of genetically modified cells with potential pain and scarring at graft sites. As an alternative, direct vector administration to skin would permit corrective gene transfer and long-term transgene expression without the complications associated with *ex vivo* therapy. We previously developed a minimal lentiviral vector to express full-length COL VII in RDEB keratinocytes and fibroblasts. In this study, we assessed the ability of lentiviral vectors to achieve COL VII gene transfer *in vivo* using an animal model. We generated and intradermally injected vesicular stomatitis virus envelop G pseudotyped lentiviral vectors encoding COL VII transgene into the skin of athymic hairless mice. Skin biopsies were obtained every week after injection and subjected to immunostaining using an antibody specific for human COL VII and the antibodies specific for cell-surface proteins to mark cell types. Immunostaining demonstrated that recombinant lentiviral vectors transduced mouse dermal fibroblasts with high efficiency. The cellular expression of COL VII remained stable *in vivo*. In contrast, very limited transduction of basal or suprabasal mouse epidermal keratinocytes was detected. Interestingly, one week after a single injection, mouse cells transduced by lentiviral vectors synthesized and deposited human COL VII within the mouse basement membrane zone. The delivered COL VII expression was confined locally at the injection sites since there was no BMZ immunostaining for human COL VII in uninjected skin sites. These data indicate that efficient and long-term *in vivo* COL VII gene transfer into skin via direct lentiviral vector injection may offer a valuable approach to the treatment of DEB.

0647

Identification of XP-C heterozygotes using real-time quantitative reverse transcriptase-PCR: a novel approach to study cancer susceptibility in XP-C gene carriers

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XP-C is an autosomal recessive disorder that requires two mutant copies of the gene to express the clinical phenotype including skin neoplasms. One study, performed before XP genes were cloned, suggested that carriers of an XP gene (estimated from pedigrees) are predisposed to skin cancer (JNCI, 62: 1415, 1979). Now that XP genes have been cloned, it should be possible to study cancer risk in known XP heterozygotes. Here we describe a test to detect XP-C heterozygotes. First, we characterized the causative mutations in the XPC gene in cell cultures from 8 patients assigned to XP-C by host cell reactivation. All mutations resulted in premature termination codons (PTC). We then confirmed that the cell lines from the parents were heterozygous for the same mutation by DNA sequencing. We developed real-time quantitative reverse transcriptase-PCR (QRT-PCR) assays to measure XPC mRNA levels (amounts of exons 4 or 12) in the cell lines. Relative to 10 normal controls the mean XPC mRNA levels were 31% (exon 4) and 23% (exon 12) in 8 XP-C cell lines. This reduced XPC mRNA from XP-C patients was anticipated from the presence of PTC that induce nonsense-mediated mRNA decay. The mean XPC mRNA levels in cell lines from 13 XP-C heterozygotes was intermediate (69% and 66% of normal). This novel approach allows the testing of individuals at risk for being XPC heterozygotes and should permit the study of a genetic predisposition to sun-induced skin cancers in XPC heterozygotes.

0648

Regulation of alternative splicing of *GLI1* in normal skin and in basal cell carcinoma

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The oncogene *GLI1*, encodes a zinc-finger transcription factor that is regulated by the Sonic hedgehog-Patched signaling pathway. Aberrant signaling of this pathway results in over-expression of GLI1, which is associated with carcinogenesis and has been found to occur in liposarcomas, astrocytomas, medulloblastomas, rhabdomyosarcomas and basal cell carcinomas (BCCs). We have previously identified two alternative *GLI1* transcripts in human tissues that differ in their 5'-untranslated regions (5'-UTRs). The longer 5'-UTR variant is formed by the splicing of exons 1 and 1A and contains multiple uATGs and uORFs, which directly result in translation repression of the main ORF. The shorter 5'-UTR, which is generated by skipping of exon 1A, lacks these features and is associated with high levels of expression of the main ORF. Notably, the longer form is the predominant transcript in adult and neonatal tissues, while the shorter form is the only variant detected in BCCs. In order to identify functional RNA *cis-elements* we generated both wild type and mutant *GLI1* pre-mRNA minigene constructs and analysed their spliced products in transfected cells. These studies identified potential splicing enhancer and silencer sequences in intron 1 and exon 1A. In parallel, we tested the ability of several splicing modulators to influence splicing decisions in co-transfection experiments. Of these only Tra2beta1, Tra2beta3 and ASF produced a significant change in the ratio of spliced products. Interestingly, Tra2beta1, which is itself an alternatively spliced product of the *Tra2beta* gene, antagonized the action of Tra2beta3 and ASF. Although, the relative activity of these splicing modulators in BCCs is not yet known, our results are consistent with other studies showing that expression of splicing factors is altered in tumour cells. These results also support our hypothesis that the large increase in GLI1 levels observed in BCCs is not only due to increased gene transcription but also to the skipping of exon 1A.

0649

Intracellular calcium regulation in SERCA2 heterozygous cells

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Darier's disease (DD) is a rare autosomal dominant disease caused by a mutation of the ATP2A2 gene, encoding the SERCA2 Ca^{2+} ATPase. The ATP2A2 is localized to the endoplasmic reticulum of cells and is important in Ca^{2+} -induced Ca^{2+} signaling, which controls essential keratinocyte functions such as differentiation, motility and cell-to-cell adhesion. Previously, we have shown that Golgi Ca^{2+} stores, controlled by a similar Ca^{2+} ATP'ase ATP2C1, are essential for normal Ca^{2+} signaling, and that mutations in the ATP2C1 cause the similar acantholytic disorder Hailey Hailey disease. To test how the ER-localized ATP2A2 mediates Ca^{2+} signaling, we used a mouse model of DD, the SERCA2+/- mouse, where one copy of the SERCA2 gene was deleted. Keratinocytes derived from SERCA2+/- mice showed distinct abnormalities in Ca^{2+} signaling, including altered baseline Ca^{2+} concentrations and impaired responses to raised extracellular Ca^{2+}. In addition, recovery rates from Ca^{2+} loads induced both by raised extracellular Ca^{2+} and release of intracellular Ca^{2+} using thapsigargin were altered, suggesting upregulation of other Ca^{2+} transport mechanisms such as intracellular ATP2C1 or plasma membrane Ca^{2+} transporters such as the plasma membrane Ca^{2+} ATPase or Na^{+}/Ca^{2+} antiporter. Capacitive Ca^{2+} influx was blunted in keratinocytes from SERCA2+/- mice, suggesting that the ATP2A2 mediates Ca^{2+} influx stimulated by intracellular Ca^{2+} store release. Finally, total cell store Ca^{2+} was decreased in SERCA2+/- cells, demonstrating that ER Ca^{2+} controlled by the ATP2A2 constitutes a major intracellular Ca^{2+} store in the keratinocyte. These experiments suggest that the Golgi and ER Ca^{2+} ATPases serve different, but coordinated functions, in controlling Ca^{2+} -induced signaling in keratinocytes. Further experiments will allow us to determine how these different Ca^{2+} signals control keratinocyte differentiation, cell-to-cell adhesion, and apoptosis.

0650

A case of malignant peripheral nerve sheath tumor with sporadic neurofibromatosis type 1 resembling a malignant fibrous histiocytoma

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A case of malignant peripheral nerve sheath tumor (MPNST) in a 46-year old female with sporadic Neurofibromatosis type 1 is reported. She had been our patient for 13 years. Physical examination showed cafe-au-lait spots, severe scoliosis and hundreds of cutaneous neurofibromas, mostly on her trunk. Fluorescence in situ hybridization using a P1-bacteriophage probe spanning exons 2-11 demonstrated a deletion of the NF1 gene. Her constitutional DNA was examined at 8 intragenic polymorphic sites and only 1 allele at each site was identified, which supports the likelihood this is a whole gene deletion. We used 30 polymorphic markers distributed across all chromosome arms to evaluate genome stability in 5 separate neurofibromas from this patient. Two neurofibromas showed allelic imbalance at 9q, whereas all other markers demonstrated bi-allelic balance. This indicates normal genomic stability in dermal neurofibromas in a deletion patient. She was admitted to our hospital with a painful mass on her right shoulder that had grown rapidly for 7 months. MRI demonstrated a large tumor in the right shoulder, and she underwent a total tumor resection. Histological examination of the tumor revealed nuclei that were variable in size and showed remarkable atypia and proliferation of spindle-shaped cells and pleomorphic areas. The tumor demonstrated a mixed pattern of typical MPNST and malignant fibrous histiocytoma. Immunohistochemically, the tumor cells were negative for S-100 protein, NSE, CD34, HMB-45 and positive for CAM5.2. After surgery, the patient presented with local recurrence and multiple pulmonary and brain metastasis. She died 26 months after diagnosis. This case supports the hypothesis that increased tumor burden predicts the possibility of a whole-gene deletion, and may increase the chance of a MPNST arising. Combining surveillance of signs and symptoms with whole-gene deletion detection screening could help in the early detection of MPNST.

0651

Genetic polymorphism of IL-12 p40 gene in the Japanese population with Behcet's disease

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Behcet's disease (BD) is a chronic inflammatory disorder characterized by oral and genital ulcerations and various cutaneous, ocular, manifestations. It has been reported that immunological abnormalities and genetic factor play a great role in its pathogenesis. However, the mechanism of them is imperfectly understood. Th1 type proinflammatory cytokines such as IL-12 and interferon-gamma are detected predominantly in the lesional skin of patients during active phase of the disease. The purpose of the study is to examine the polymorphism of IL-12 p40 gene in BD. Previous reports of single nucleotide polymorphism (SNP) in the IL-12 p40 gene revealed that the most common SNP was position 1188 in the 3' untranslated region (UTR) in several autoimmune diseases. We studied IL-12 p40 gene polymorphism of 17 Japanese with BD and 63 Japanese healthy volunteers. Genomic DNA was obtained and was amplified by polymerase chain reaction (RCR). PCR products were digested with TaqI and restriction fragment length polymorphism (RFLP) method was performed. We revealed that allele A frequency of BD patients and healthy controls were 0.559 and 0.492, respectively, and that C frequency of BD and healthy was 0.441 and 0.508, respectively. These were no differences of IL-12 p40 gene in 1188 genotype between BD and healthy controls. However, AA genotype tended to be decreased in BD, compared to healthy controls. These date suggest that IL-12 p40 gene is susceptible in BD in terms of SNP analysis, although further analysis should be necessary to conclude this.

0652

Alteration of the epidermis of patients homozygous and heterozygous for a mutation in connexin 26

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Gap-junctions (GJ) are cell-cell junctions important for direct communication and coordination of adjacent cells by forming selective channels for small molecules, e.g. second messengers. They play important roles in proliferation, migration, and differentiation of many cell types. GJ consist of connexins (Cx), a growing family of membrane-spanning proteins. Mutations in Cx 26 have been shown to result in deafness. Recently we demonstrated an increased thickness of the epidermis of Ghanaian patients homozygous and heterozygous for the mutation R143W in Cx 26 and discussed an evolutionary advantage of carriers of this mutation. Here we show the molecular details of the epidermis of these patients by using immunohistochemistry and demonstrate alterations of the synthesis and distribution of barrier-forming and differentiation specific proteins, i.e. cornified envelope proteins, tight junction proteins and cytokeratins, in the skin of heterozygous and homozygous carriers of the mutation. The possible roles of these alterations for a heterozygous advantage are discussed.

0653

Genetic correction of DNA repair deficient/cancer prone xeroderma pigmentosum group C keratinocytes

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Xeroderma pigmentosum (XP) is a rare photosensitive and cancer prone syndrome transmitted as an autosomal recessive trait. Most of the cancers developed by XP patients are basal- and squamous cell- carcinomas strikingly restricted to sun-exposed parts of the skin. Cells from most classical XP patients are deficient in nucleotide excision repair, a versatile biochemical mechanism for removal of ultraviolet-induced DNA lesions. Among the seven classical XP complementation groups known to date (XP-A to XP-G), XP-C is the most common one in Europe and North Africa. We have undertaken genetic correction of XP-C fibroblasts and particularly keratinocytes, which are most relevant cells in relation to skin cancer and, have proven recently to be capable of reconstructing XP-C skin in vitro. Primary keratinocytes from XP-C patients were transduced with a retroviral vector stably driving the expression of the wild-type XPC protein. These genetically corrected XP-C keratinocytes recover normal levels of DNA repair capacity, cell survival and clonal transition toward UVB irradiation. We also show that, in the absence of UV, XP-C keratinocytes exhibit intrinsic cell cycles abnormalities, and b-1 integrin overexpression, defects that are also both fully reversed after genetic correction. Finally, in vitro reconstruction of skin from genetically corrected XP-C keratinocytes opens the first rational perspective to XP tissue therapy.

0654

Genetic analysis of PSORS1 single nucleotide polymorphisms (SNPs) in late onset, guttate and palmoplantar pustular psoriasis

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The major susceptibility locus for psoriasis vulgaris (PV) is a 200kb region (PSORS1) within the MHC on chromosome 6p21.3. Candidate genes in this region including HLA-C, HCR and CDSN show association with familial (early onset) PV. Late onset psoriasis (LOP) is defined by disease onset post 40 years of age and is sporadic. Guttate psoriasis (GP) and palmoplantar pustulosis (PPP) are distinct clinical subtypes of psoriasis and may occur concurrently with PV. Our aim was to assess the role the PSORS1 locus in LOP, GP and PPP. Genotyping for HLA-C alleles and a total of 19 SNPS located within PSORS1 was performed on LOP (n = 150), GP (n=137), PPP (n=106) and controls (n = 309). Statistical analysis was by the Chi-squared test. LOP demonstrated limited association to PSORS1 genes ie HLA-Cw6 ($p = 0.001$), CDSN*5 ($p = 0.013$) and HCR 325 ($p = 0.023$). In contrast, GP showed very strong association with HLA-Cw6 ($p = 4 \times 10^{-40}$), CDSN 1243 ($p = 4 \times 10^{-7}$) and HCR SNPs (eg HCR 325 $p = 2 \times 10^{-21}$). Predicted haplotypes from population data (PHASE 1.0) showed that these GP alleles exist on a similar risk haplotype to that recently reported for PV. PPP showed no association with these genes and no correlation with age of onset for disease was observed. Thus GP is almost exclusively associated with the PSORS1 risk haplotype, indicating a common genetic basis for susceptibility to GP and PV. Molecular studies of GP are therefore likely to be highly informative. The LOP group may contain a minority of individuals with early onset-type disease hence the limited association with PSORS1 SNPs. For future LOP studies PSORS1 association may be helpful as an exclusion criterion. PPP appears to have a different genetic aetiology.

0655

Comprehensive and systematic analysis of function and expression of all the 51 missense mutants of sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b found in Darier's disease patients

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Darier's disease (DD) is caused by mutations in endoplasmic reticulum Ca^{2+}-ATPase 2b protein (SERCA2b)[1)] responsible for Ca^{2+} uptake coupled with ATP hydrolysis and hence Ca^{2+} homeostasis in skin cells. To reveal consequences of the mutations and thus to understand the mechanism for DD, we introduced all of each 51 missense mutations so far reported with DD pedigrees into SERCA2b cDNA and expressed the protein in COS-1 cells. The expression level and function (the specific ATPase and Ca^{2+} uptake activities) of the expressed mutants were determined with microsomes prepared from the cells, and compared with those of the wild type. The expression level and specific ATPase activity varied dramatically depending on the mutations introduced. In 33 mutants among 51, the activity was almost completely lost regardless of their expression level. In contrast, the other 18 mutants showed fairly high activity (80-40% of that of wild type) and also high (only moderately reduced) expression level. Some of these mutants possessed no Ca^{2+} uptake activity in spite of high ATPase activity, thus being non-functional (the "uncoupled" mutants), but others possessed the "coupled" Ca^{2+} uptake activity. Results indicated that not only complete loss of function and expression but also slight or moderate reduction in the function and expression of SERCA2b will likely result in severe changes in Ca^{2+} homeostasis and thus cause DD. Importantly, results further suggested that possible improvement in protein stability and processing during expression in some mutants may increase both their expression level and the active fraction of the expressed mutants, thus possibly lead to normal Ca^{2+} homeostasis. Kinetic and physical properties of all the mutants and their processing in the cells are now being further explored.

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Identification of DLx3 interacting factors using a retroviral system and mass spectrometry analysis

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Dlx3 is a homeodomain protein that is essential in murine skin differentiation and early development. Mutations in the human DLX3 gene result in a truncated protein that is associated with the autosomal inherited human Tricho-Dento-Osseous (TDO) syndrome. In order to identify Dlx3 cofactors or binding proteins, a retrovirus expression system was used to generate a stable Dlx3 transduced HeLa cell line. Cells were transduced with a recombinant retrovirus expressing a bicistronic mRNA encoding FLAG-Dlx3 linked to an IL2R subunit surface marker. The Dlx3 and Dlx3TDO coding sequences were subcloned to generate FLAG-HA-Dlx3 or FLAG-HA-Dlx3TDO as N-terminal tagged proteins. The detection of the IL2R on the surface of the transduced cells was used to sort out expressing cells using magnets and Dynalbead-anti IL2R magnetic beads. The sorted cells were expanded and nuclear extracts were prepared from recombinant HeLa and control HeLa cells, and protein complexes were purified using consecutive anti-Flag and anti-HA affinity columns. The affinity purified protein complexes were separated on SDS-PAGE gel electrophoresis. Coomassie blue-stained protein bands on SDS-PAGE gels that are specifically observed only in the Dlx3 transduced and Dlx3TDO samples will be excised, destained, digested with trypsin. Direct identification of the specific interacting proteins will be performed by mass spectrometry. MS peptide mapping and MS/MS sequence will be used to search the protein and EST databases for protein identification. The results of potential interacting proteins that associate with Dlx3 or Dlx3TDO, and their roles in transcriptional activation will be reported.

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PSORS1C3, a new gene in the PSORS1 region on chromosome 6p21.3

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The psoriasis susceptibility locus PSORS1 on chromosome 6p21.3 has under the last few years been under thorough examination in the search for a major genetic factor underlying the cause of psoriasis. Several putative candidate genes have been investigated but the strongest association in this region shown to date is still that of the HLA-C allele Cw*0602. However, controversy surrounds whether this is a true psoriasis gene or a marker in linkage disequilibrium with the PSORS1 gene. Therefore the search continues. Although the MHC region is one of the most gene dense regions in our genome the area surrounding HLA-C is bereft of genes. We therefore concentrated our efforts in searching for new possible candidates at this locus. Here we present PSORS1C3 (Psoriasis Susceptibility 1 Candidate 3), a newly cloned gene located telomeric to HLA-C in the minimum risk region. We have characterized this gene with regard to psoriasis involvement in the Swedish population. PSORS1C3 is composed of three exons and stretches a 4.4 kb region in a centromeric to telomeric direction. From Northern blot analysis we have found PSORS1C3 to be expressed in various tissues including normal and psoriatic skin, and 3' and 5'RACE have been used in order to confirm the size. We amplified all exons in 3 PCR fragments and sequenced from both ends in a series of Swedish psoriasis individuals, while comparing them to healthy population matched controls. Assembly of resulting sequences was performed in the Staden sequence assembly package and polymorphisms were identified using the Trace-Diff function. Using this association analysis we have found significant association to psoriasis by single nucleotide polymorphisms in two of the exons. This association could be explained by linkage disequilibrium with Cw*0602 however, the biological function of this gene is still unknown and hence variations within it may have a biological significance.

0658

Induced apoptosis, and altered adhesion, proliferation and gene expression of keratinocytes in the epidermis of mice deficient in the laminin γ2 chain

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Junctional epidermolysis bullosa (JEB) is associated with mutations in six distinct genes expressed in the cutaneous BMZ; these include LAMA3, LAMB3 and LAMC2 which encode laminin 5 subunit polypeptides. Here we generated a mouse model for JEB by inactivating the laminin γ2-chain gene by targeted frameshift deletion of exon 8 in Lamc2. Heterozygous mice were phenotypically normal, while $Lamc2^{-/-}$ mice showed blistering phenotype on day 1-2 of neonatal period, and died within 5 days of birth. The $Lamc2^{-/-}$ mice demonstrated absent expression of laminin γ2-chain on the BMZ and attenuated expression of α3 and β3-chains of laminin. Transmission electronmicroscopy revealed rudimentary, poorly developed hemidesmosomes. Epidermis of the $Lamc2^{-/-}$ mice revealed induced apoptosis in the basal cells of the blistered skin, suggesting that cell-matrix adhesion provided by laminin 5 plays a role in cell survival *in vivo*. The targeted mutation was shown by RT-PCR to result in two transcripts *in vivo*, harboring the deletion of either exon 8 or exons 8 and 9 combined, and resulting in premature termination codons in exon 9 or 10, respectively. Thus, the targeted mutation resulted uniformly in null-allele *in vivo*. The cultured $Lamc2^{-/-}$ keratinocytes demonstrated slightly positive staining with γ2-chain specific antibodies, which could be explained by the presence of a transcript with partial restoration of the reading frame due to alternative splicing *in vitro*. Nevertheless, primary keratinocytes cultured from $Lamc2^{-/-}$ mice proliferated in different matrices, including plastic, type IV collagen, laminin and Matrigel, and attached to type IV collagen and Matrigel as efficiently as the wild type keratinocytes, while their attachment on plastic and laminin was significantly weaker. In summary, $Lamc2^{-/-}$ mouse recapitulates human JEB and provides novel insight into the role of laminin 5 in keratinocyte biology.

0659

Identification of a receptor-selective, ligand-dependent nuclear receptor coregulator from epidermal keratinocytes

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Keratinocyte (KC) response to peroxisome proliferators (PPs) is complex with ultimate effects on differentiation-associated gene expression and in some cases cell replication. Part of this multi-faceted response to PPs may have to do with the availability of different PP activated receptor (PPAR) protein coregulators at different stages of KC maturation. We have recently extended our investigation of KC response to PPs to include analysis of coregulator expression as well as isolation and characterization of potentially epidermal-specific PPAR coactivators/corepressors. Using the carboxyl terminal portion of PPARalpha in a yeast-two-hybrid screening of a human KC cDNA library, a new coregulator was isolated. This clone has partial homology to a previously identified PPAR coregulator and appears to be an isoform derived from alternative splicing suggesting yet another means of controlling coregulator function. Relative amounts of the isoform transcripts vary in different epithelial and non-epithelial tissues. Functionally, in activating gene expression the variants differ in their ability to cooperate with PPAR and other nuclear receptors relevant to KCs. These activities are however dependent on the reciprocal interaction of hydrophobic amino acid motifs in the coregulator and nuclear receptor. Notably, while relatively small (predicted mass of 26.9 and 32.5kD) compared to other nuclear receptor coregulators, these proteins possess intrinsic activating functions that contribute to their regulation on nuclear receptor-mediated transcription. Additionally there is coregulator interaction domain beyond the hydrophobic motif allowing for further protein-protein interactions in the transcription complex. We expect defining the repertoire of coregulators present in keratinocytes will improve our understanding PPAR function in general and the mechanism of the epidermal response to PPs in particular.

0660

The clinical spectrum of Keratitis-Ichthyosis-Deafness syndrome

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Keratitis-Ichthyosis-Deafness syndrome (KID) is an ectodermal dysplasia due to dominant mutations in GJB2 encoding the gap junction protein Cx26. To delineate the clinical spectrum of this disorder, we evaluated 7 patients with sporadic KID between 3 to 56 years of age. The most consistent features observed were sensorineural hearing loss (SNHL), palmoplantar keratoderma, cheilitis/perleche, skin infections, and follicular hyperkeratoses. SNHL was bilateral in 6 of the 7 patients and ranged in severity from moderate (2), to severe (2), to profound (4). All but the youngest patient had corneal neovascularization and in 5 patients it was accompanied by keratitis and/or photophobia. We noted a marked variability of the cutaneous phenotype. A subset of 3 patients had sharply demarcated, figurate outlined, erythematous and hyperkeratotic plaques on the face and limbs. Four patients demonstrated no erythema and presented with spiculate follicular hyperkeratoses, diffuse, acanthotic thickening of the skin, or both. The features of one child were distinctive and overlapped with Clouston syndrome, including atrichia, nail dystrophy, and facial dysmorphism. During puberty, 2 males of this group and 1 female progressively developed a follicular occlusion syndrome. They had pustules, large inflammatory cysts, multiple nodules and draining sinuses of the scalp leading to scarring alopecia. This clinical presentation is suggestive of an underlying defect in keratinization of the hair follicle. The 3 oldest patients of our cohort had a history of mucocutaneous squamous cell carcinoma developing between the ages of 38 to 53. This high incidence illustrates the need for careful cancer screening in these patients and underscores the specific role of Cx26 in carcinogenesis that is not yet fully understood.

0661

Genetics of alopecia areata using a mouse model

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Alopecia areata (AA) is an autoimmune disease that targets actively growing (anagen) hair follicles in humans, mice, rats, dogs, horses, and cattle. C3H/HeJ mice spontaneously develop AA from 5 months of age and older in females and later in males. Frequency of AA approached 20% in a colony by 18 months of age. C57BL/6J mice do not develop AA. A segregating F2 population of female mice (n=1096) was generated from crossing these two strains. AA (n=138) and clinically normal (n=214) mice were genotyped at 12 months of age using 211 microsatellite probes. The peak LOD score on mouse Chromosome 17 (10.9) was around marker D17Mit134 at 16.9 cM from the centromere. The mouse histocompatibility locus, H2, the mouse equivalent of HLA in humans, was a likely candidate. Twelve month old C3H.SW-H2b/SnJ mice (C3H/HeJ congenic mice in which the H2k purported susceptibility locus was replaced with the H2b purported resistance locus) did not develop AA supporting this locus as being important in AA. A suggestive linkage was also found on mouse Chromosome 9 (LOD score 2.0) around D9Mit206, 20 cM from the centromere. The interval on mouse Chromosome 17 contains several orthologous genes potentially associated with human AA.

0662

Novel mechanism of revertant mosaicism in Dowling-Meara epidermolysis bullosa simplex

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The severe Dowling-Meara form of epidermolysis bullosa simplex (EBS-DM) is caused by dominant-negative mutations in keratins K5 and K14, which are specifically expressed in the basal keratinocytes of the epidermis. The most common mutation in EBS-DM patients is the missense mutation R125C in exon 1 of the K14 gene. Here, as part of an on-going gene therapy initiative, we made keratinocyte cell line from a sporadic case known to carry the R125C mutation. This female patient was in her early 20s and had suffered from widespread herpetiform blistering since birth, typical of EBS-DM. A skin biopsy was taken from a blister-free site on the lower back, from which primary keratinocytes were cultured. As a routine check, the full-length K14 cDNA was sequenced using keratinocyte mRNA. Surprisingly, the R125C mutation was barely detectable in keratinocyte cDNA whereas it was present in a 1:1 ratio with the normal allele in lymphocyte DNA. Also, in the cDNA sequence, very low levels of overlapping sequence traces could be seen upstream of the R125C mutation. These overlapping traces were clearly visible when genomic DNA derived from the keratinocyte cultures was sequenced. This was found to be a heterozygous 1 bp insertion mutation, designated 242insG, which creates a premature termination codon immediately downstream. The insertion was completely absent in lymphocyte DNA. Cloning of the keratinocyte genomic PCR products revealed that the R125C allele also carried the 242insG frameshift. Thus, in this non-lesional area of the patient's epidermis, the allele carrying the dominant-negative mutation has been silenced by the occurrence of a second, nullifying, mutation. Immunofluorescence staining of the primary cultures with a K14 antibody showed that only a small percentage of cells had filament aggregates, presumably cells lacking the revertant mutation. This case represents a novel mechanism of revertant mosaicism with implications for gene therapy.

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CCL18 identified in CD1a+ cells of Langerhans cell histiocytosis by suppression subtractive hybridisation

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The purpose of this study was to identify differentially expressed genes in purified CD1a+ cells isolated from Langerhans Cell Histiocytosis (LCH) lesions. Suppression subtractive hybridisation allows for the generation of a disease specific cDNA library from as little as 0.05 µg of total RNA and normalizes the relative abundance of cDNA species during hybridisation, increasing the sensitivity of this methodology and allowing identification of rare mRNA transcripts not identifiable by other methods. Dermal nodule and donor skin were decontaminated in RPMI 1640 medium containing penicillin, streptomycin and amphotericin B for 30 minutes and the epidermis and dermis separated after dispase digestion. Epidermal and dermal cell suspensions were formed by syringe disruption of trypsin digested epidermis and sieve disruption of collagenase digested dermis. Cell suspensions were washed and the Langerhans cells (LC) or LCH cells were purified using mouse anti-human CD1a (NA1/34) antibody and anti-mouse immunoglobuln coatd Dynal beads. Cytoplasmic RNA was purified by cell lysis and guanidine isothiocyanate/phenol/chloroform extraction. Pooled RNA from seven donors was used as driver to reduce major histiocompatibility differences. The Smart PCR cDNA synthesis kit (BD Clontech) was used to selectively reverse transcribe poly (A)+ RNA and the resulting cDNA used to generate a suppression subtractive library using the PCR-select cDNA subtraction and TOPO TA cloning Kits (BD Clontech and Invitrogen; respectively). Colonies containing differentially expressed gene products were identified by cDNA array, sequenced and sequences identified using the Blast program. Colony 50585 failed to hybridise to reverse subtracted or LC probes, but showed strong hybridisation to LCH probes and increased hybridisation to forward subtracted probes. Sequencing of this plasmid identified an insert 168 bases in length which was matched by Blast analysis to the chemokine CCL18. We have demonstrated the expression of CCL18 in LCH CD1a+ cells and the lack of expression in normal LC.

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Interleukin-20 is up-regulated in psoriasis and responds to conventional and novel biologic therapy

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IL-20, a new member of the IL-10 family of cytokines, has been implicated in psoriasis on the basis of observed increases in IL-20R mRNA in lesional skin and the presence of epidermal hyperplasia in transgenic mice overexpressing IL-20. To further investigate the role of IL-20, we used real-time PCR to quantify mRNA levels for IL-20 cytokine, IL-20Rα, and IL-20Rβ in skin extracts. In 19/19 psoriatic patients, IL-20 mRNA levels were elevated when we compared matched pairs of non-lesional (NL) and lesional (PS) skin. Mean expression (normalized to the housekeeping gene hARP) was 36.2 for NL and 948.9 for PS skin (expression was 26-fold higher on average in PS skin, $p<0.0001$). Furthermore, when epidermis was split from dermis in PS biopsies, most of the IL-20 mRNA (>90%) was found by RT-PCR to be localized to the epidermis. We also found IL-20Rα and IL-20Rβ mRNA decreased in PS compared to NL skin. Mean expression of IL-20Ra was 39.7 in PS skin compared to 57.4 in NL skin ($p=0.047$), while mean expression of IL-20Rβ was 3.42 in PS skin compared to 4.98 in NL skin ($p=0.015$). Expression of IL-20Rα and IL-20Rβ in skin of unaffected controls was greater than that in psoriatic lesions (in contrast to results in published reports) and overall similar to levels in NL skin. Following various forms of therapy, IL-20 mRNA levels were found to return to those seen in NL skin. In lesional skin, IL-20 mRNA levels normalized after therapy with NB-UVB light (80% decrease, $p=0.035$), as well as alefacept (82% decrease, $p=0.004$). Considering that alefacept specifically targets T-cells, these data argue that T-cells are proximate regulators of increased IL-20 expression in psoriasis.

0665

N-terminal deletion of laminin A3A isoform causes the chronic granulation tissue disorder LOC syndrome

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Laryngo-onycho-cutaneous (LOC or Shabbir) syndrome (OMIM 245660) is an autosomal recessive disorder characterised by cutaneous erosions, nail dystrophy and excessive vascular granulation tissue, particularly in the conjunctiva and larynx. Although this syndrome has some features in common with epidermolysis bullosa (EB), skin blistering is not a clinical feature and LOC only occurs in the Punjabi Muslim population. Ultrastructural and immunohistochemical assessment of skin showed no consistent abnormalities. Genomewide homozygosity mapping using consanguineous families localised the gene to a 500 kb region on chromosome 18q11.2 with a LOD score of 19.8. This region contained the laminin alpha-3 gene (LAMA3) in which homozygous null-alleles cause the lethal Herlitz form of junctional EB. Detailed investigation of the locus revealed 2 novel transcripts (LAMA3B2 and LAMA3C) in addition to the known LAMA3A and LAMA3B isoforms. The complete LAMA3 gene was shown to consist of 77 exons spanning more than 320 kb of genomic DNA. The mutation in all LOC families was found to be a frameshift in exon A1 (118insG), specific to the LAMA3A isoform. Interestingly, the mutant transcript is rescued from nonsense-mediated decay by use of an alternative translation start site, therefore leading to an unusual N-terminal deletion of LAMA3A. This data points to a hitherto unknown role for the LAMA3A N-terminus in control of granulation tissue formation in LOC patients and perhaps in other disorders associated with abnormal wound healing.

0666

Genotype-phenotype correlations in KID syndrome: novel GJB2 mutations and association with squamous cell carcinoma

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Keratitis-Ichthyosis-Deafness syndrome (KID) is a rare ectodermal dysplasia affecting multiple stratifying epithelia resulting from dominant mutations in GJB2, encoding the gap junction protein Cx26. To explore the spectrum of mutations and extend genotype-phenotype correlations of this clinically heterogeneous disorder, we screened GJB2 in 5 unrelated patients with sporadic KID syndrome. All had hyperkeratosis of the skin, sensorineural hearing loss, and with one exception, corneal vascularization and palmoplantar keratoderma. Sequence analysis revealed in each patient a missense mutation of GJB2. We identified 2 novel mutations in the amino-terminus (NT) of Cx26 as well as the common D50N mutation. One patient was found to be homozygous for a G to A transition not present in the mother's DNA. Haplotype analysis excluded paternal isodisomy of ch. 13, suggesting the presence of a GJB2 deletion on one allele. Two patients of our cohort developed squamous cell carcinoma at sites of hyperkeratotic, and chronically infected plaques on the legs. Nevertheless, other cutaneous features differed, including symmetrical plaques of erythrokeratoderma on face and extremities, or pustules, cysts, draining sinuses and deep scars consistent with follicular occlusion syndrome. Both individuals carried different GJB2 mutations, including D50N and a novel missense mutation changing the charge of the NT domain. Taken with the previously documented mutations, our results indicate that codon 50 in the first extracellular domain of Cx26 is a mutational hot spot, while other mutations cluster in the cytoplasmic NT. Patients carrying D50N and other KID mutations are at high risk for squamous cell carcinoma, yet the specific mechanisms by which faulty Cx26 leads to tumorigenesis remain to be elucidated.

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Epidermal compartments display different patterns of expression of a secreted gene product in an *in vivo* gene delivery model

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The epidermis is an attractive target tissue for expressing genes that give rise to a secreted product. In vivo delivery of naked plasmid DNA represents one approach for given applications of cutaneous gene therapy. We speculated that different epidermal compartments may show varied patterns of transgene expression using this approach. To test this hypothesis, we delivered a secreted alkaline phosphatase (SEAP) marker gene under control of either the CMV, involucrin (inv), or keratin 14 (k14) promoters to mouse skin using gene gun-mediated DNA transfer. Five adult CD-1 mice were included in each group. Each construct was delivered to 8 sites (1 μg/site) of depilated back skin under 400 psi of pressure. Blood samples were obtained from each mouse prior to gene delivery and at days 1, 2, 4, and 7 after treatment. SEAP levels were measured using a chemilumenesence assay. Mice receiving a SEAP transgene under control of either the CMV or inv promoters typically showed peak SEAP levels at day 1, although peak levels were about 2.5 fold greater for CMV mice (27.3 ng/ml) compared to inv mice (11.1 ng/ml). By day 7, SEAP levels declined to near background for inv mice (0.36 ng/ml), although higher SEAP levels were observed for CMV mice (1.66 ng/ml). In contrast, k14 mice showed substantially lower peak SEAP levels (3.0 ng/ml), and these were observed at day 4. Day 7 levels (1.6 ng/ml) were comparable to day 1 levels (1.8 ng/ml). Increasing number of sites and amount of DNA per site yielded higher blood levels of SEAP. X-gal staining of skin receiving a lacZ gene confirmed expression was limited to epidermis and hair follicles for all promoters. Although factors such as promoter strength and efficiency of gene delivery to different compartments may influence overall expression levels, these results suggest transient expression directed to basal keratinocytes may be more sustained and consistent compared to suprabasal keratinocytes. Studies with alternative compartment-specific promoters may help further substantiate these results.

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Sustainable corrective TGM1 gene delivery in human lamellar ichthyosis patient skin in vivo using integrating non-viral plasmid gene transfer

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Genetic correction of genodermatoses represents a potentially effective molecular therapy, however, the viral vectors comprising the mainstay of such efforts suffer from major biosafety and cost concerns. Recent development of nonviral approaches to integrate plasmid DNA, such as via the phiC31 integrase which has been recently studied in epidermolysis bullosa, offer an attractive alternative to viral vectors. Integrating plasmids overcome the prior difficulties with transience of gene delivery seen with episomal plasmids, which are rapidly lost in self-renewing tissues such as epidermis. To test the ability of this strategy to stably correct the human genetic skin disease, lamellar ichthyosis (LI), we generated an attB sequence-containing phiC31 integrase transfer vector encoding transglutaminase 1 (TGase1). After gene transfer in vitro, primary LI keratinocytes were used to regenerate skin tissue on immune deficient mice [n=5 mice per vector group]. TGase1 expression was sustained in vivo by this approach out to 5 months post-grafting, the duration of the experiment. In addition, corrected tissue demonstrated smooth surface texture and normalization of barrier function, as measured by transepidermal water loss (TEWL). In contrast, mock-transfected LI controls failed to demonstrate any detectable TGase1 expression as expected and grafts failed after 1 month. Further, uncorrected skin tissue was hyperkeratotic and displayed markedly increased TEWL. These data confirm that durable gene delivery spanning multiple epidermal turnover cycles can be achieved in multiple human genetic skin disorders using non-viral gene transfer, suggesting that integrating plasmids may provide a useful broad plaform for future cutaneous gene therapy efforts.

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A scoring system for predicting CDKN2A status in a population-based melanoma clinic

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With the availability of commercial CDKN2A testing, a simple and reproducible method of identifying potential carriers in clinical practice is essential. To this end, we devised a simple scoring system to evaluate CDKN2A carrier risk. We have prospectively enrolled 88 probands (from 88 independent families) who had a personal history of melanoma and (i) one other 1st-degree relative with melanoma (ii) two or more relatives with melanoma or (iii) three or more multiple primary melanomas. The extant literature suggests that multiple primary melanomas, number of affected relatives in a kindred and age of onset prior to 35 are all factors related to CDKN2A mutation carrier status. In order to accommodate these variables into a single instrument, we assigned 1 point for each primary melanoma diagnosed in the proband, 1 point for each additional family member with melanoma and 1 point for a diagnosis prior to age 35. A cumulative score was then assigned to each of the probands. We then analyzed CDKN2A exons 1α and 2 by direct sequencing and exon 1β by PCR-SSCP. Twelve sequence variants (12/88 or 14%) were detected including 2 novel germline mutations (Ala60Thr; Met53Val). There were 36 probands with a score of 2, 26 probands with a score of 3, 16 probands with a score of 4 and 10 probands with a score of ≥5. The rates of germline CDKN2A variants were 8%, 7.7%, 12.5% and 50% for individuals with scores of 2, 3, 4 and ≥5, respectively. Known deleterious mutations occurred only in probands with a score of ≥4. The recurrent 24-bp duplication was seen in 3 low-score probands suggesting that it is a lower-risk allele; this finding is compatible with data that have failed to prove a functional consequence for the 24-bp duplication. In summary, we devised the first iteration of a simple instrument to predict who is at risk for harboring CDKN2A mutations and who may be appropriate for genetic testing. Furthermore, our scoring scheme may also predict the potential likelihood that a sequence variant actually represents a deleterious mutation.

0670

VDR coactivator complexes involved in 1,25(OH)2D3 regulation in normal and transformed keratinocytes

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Cell programs such as proliferation and differentiation involve the sequential activation and repression of gene expression. Vitamin D, via its active metabolite 1,25(OH)2D3, controls the proliferation and differentiation of keratinocytes by directly regulating gene transcription. We have shown that two classes of coactivator complexes, DRIP (the vitamin D receptor interacting proteins)/Mediator and the p160 steroid receptor coactivator family (SRC), are sequentially utilized during normal differentiation. Using GST-VDR affinity beads, VDR binding complexes were purified from keratinocytes and identified by mass spectrometry (MS). The multi-protein DRIP complex was identified from normal proliferating keratinocytes. Following differentiation, SRC family members replaced major DRIP proteins. Nuclear extracts from squamous cell carcinoma (SCC) showed a similar DRIP complex as proliferating keratinocytes. Detailed analysis using tandem MS peptide sequencing of the complex from normal keratinocytes revealed the presence of heat shock protein (hsp) in addition to the DRIP complex. The hsp have been implicated in active dis-assembly of steroid hormone receptor-coactivator complexes. Differential expression of DRIP205, SRC-2 and 3 was also observed. Western analysis using cultured normal keratinocytes showed that DRIP205 decreases, whereas SRC-3 increases during differentiation. In contrast, both DRIP205 and SRC-3 were over-expressed in SCC. These results were consistent with immuno-staining of DRIP205 and SRC in sections of normal skin and SCC tumors. These results suggest that VDR coactivators of DRIP and SRC are sequentially involved in VDR transactivation during normal differentiation, but this transition fails in transformed cells probably because of lack of critical factors involved in the disassembly of such complexes.

0671

Cell cycle regulation of the CYLD tumor suppressor

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CYLD is a novel tumor suppressor gene that was discovered by positional cloning of the linkage interval for familial cylindromatosis (FC), also known as Brooke-Spiegler syndrome or turban tumor syndrome. FC is an autosomal dominantly inherited disease characterized by a variety of skin appendage tumors. Germline mutations in CYLD have been demonstrated in families with FC, and loss of heterozygosity at the CYLD locus has been found in these neoplasms, suggesting that CYLD functions as a tumor suppressor. However, at the molecular and cellular level, the mechanism (s) of CYLD tumor suppression remains largely unknown. We expressed two distinct segments of CYLD in E.coli as fusion proteins with GST. Each fusion protein was purified and used as an immunogen to generate polyclonal antibodies. The antibodies were then purified using affinity chromatography and used for immunoblotting and immunofluorescence studies. We first demonstrated that the CYLD protein is expressed in the nuclear and cytoplasmic compartments in cells. Immunofluorescence studies showed punctate nuclear staining pattern in some, but not all, interphase cells. Therefore, we synchronized T24 bladder carcinoma cells at G0 by contact inhibition and HeLa cells during the M phase with nocodazole. These studies showed that CYLD resides within the cell nuclei and its expression remains low or undetectable just prior to the G1/S transition. As S phase begins, the CYLD expression increases dramatically and the protein aggregates into nuclear speckles as observed by immunofluorescence microscopy, suggesting that CYLD may be cell cycle regulated, possibly during the S-phase progression. Thus, our findings provide the first evidence that CYLD is regulated in a cell cycle specific manner, and suggest that cell cycle regulation of CYLD may be important in CYLD-mediated tumorigenesis.

0672

Pseudoxanthoma elasticum--development of a mouse model by targeted ablation of ABCC6

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Pseudoxanthoma elasticum (PXE) is characterized by calcification of pleiotropic elastic structures in the skin, eyes, and the cardiovascular system. The mutated gene was recently shown to be ABCC6 encoding MRP6, a transmembrane protein of unknown function, expressed primarily in the liver. We have embarked on development of a mouse model for PXE using targeted ablation of the ABCC6 gene. The targeting construct replaced 6.9 kb of genomic sequence resulting in elimination of exons 15-18 from the corresponding mRNA (545 bp), which is out-of-frame predicting a null allele. Southern analysis and genomic PCR showed the final products to be homozygous for the deletion in the ABCC6 gene. Northern analysis of polyA$^+$-RNA from the liver revealed that the mutant ABCC6 mRNA was not expressed, suggesting that it is unstable. Staining of liver from wild-type mice with antibody anti-MRP6 antibody revealed membrane-associated staining, while the staining was entirely negative in the ABCC6$^{-/-}$ mice. These findings confirm that the mice are truly null with regard to MRP6 expression. Examination of elastic structures by histochemical stain (Verhoeff-van Gieson) and by immunohistochemistry with anti-elastin antibody revealed that the elastic structures in the eye (Bruch's membrane), in the skin, and in the aorta were apparently normal in ABCC6$^{-/-}$ mice at eight weeks of age, and von Kossa staining for calcium was negative. These findings suggest that the development of the elastic fiber network in tissues affected in PXE is normal in these mice, at least during the early stages of development. Thus, the findings in the ABCC6 knock-out mice are consistent with late-onset of human PXE.

0673

Splicing in humans: exceptions from the rules

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Alternative splicing is an important yet little understood mechanism, which enables protein diversity by providing multiple transcripts and therefore several proteins encoded by a single gene. Bioinformatics assume that 33-59% of human genes have at least two splice variants. This explains the large number of estimated 150,000 proteins in contrast to only approximately 30,000 genes in the human genome. The highly conserved consensus sequence (AGgt) at the EXON/intron border is essential for correct splicing as it is targeted and cleaved by the spliceosome. We report a patient suffering from junctional epidermolysis bullosa, an inherited skin blistering disease, which in this case is caused by aberrant splicing in combination with a nonsense mutation (R635X) in the gene coding for the β-chain of the laminin-5 protein. Reduced protein expression was shown by almost complete lack of immuno reaction with the anti-kalinin antibody pAK4101 unsing immunoelectron microscopy. A single basepair substitution (3009CØT) in exon 20 changes the sequence AGGC to AGGT, which serves as a new splice site. Analysis of patient′s RNA revealed that the spliceosome recognizes the newly introduced exonic splice site preferentially over the original one farther downstream. This splice variant is not predicted by the common splice site scoring system of Shapiro and Senapy, which deduces the splicing probability from given RNA sequences. Although the newly introduced consensus sequence shows a score of only 68.6 in contrast to 92.2 of the original one, the mutated sequence is easily recognized by the spliceosome. These results represent an exception from the well-known provisions of splicing.

0674

The molecular role of cyclin-dependent kinase inhibitor 2A in the familial melanoma syndrome

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Germline mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A, p16, INK4a) are found in 20-40% of melanoma kindreds, making it the major melanoma predisposition gene. It is well-established that CDKN2A negatively regulates entry into the cell cycle through inhibition of the cyclin D1-cyclin-dependent kinase 4/6 complex. However, despite these scientific insights, the molecular mechanisms by which a CDKN2A mutation preferentially induces melanocytic tumors of the skin is poorly understood. A large Utah melanoma kindred carrying a temperature-sensitive germline CDKN2A mutation was utilized to test the hypothesis that primary cultures of nevus-derived cells, fibroblasts, and lymphoblasts derived from carriers and non-carriers of this mutation would demonstrate temperature- and tissue-dependent CDKN2A expression changes. Primary cultures of nevus-derived cells were grown at 34*C, 37*C, and 40*C and analyzed by Western blot, fluorescence-activated cell sorting, and cDNA microarray. Carrier-derived cells demonstrated a baseline and temperature-dependent elevation of total and phosphorylated Rb (S780) relative to non-carrier-derived cells. Cell-cycle analysis also demonstrated a shift into G2/M phases of the cell cycle in carrier-derived cells relative to non-carrier-derived cells. Expression analysis identified 30 genes that showed a significant difference in expression (ANOVA $p < 0.001$) when compared at 34*C and 40*C in carrier and non-carrier samples. Several of these genes are not known to be downstream targets in the CDKN2A pathway. A structural and functional comparison of the differentially-regulated genes will be presented.

0675

Increased expression of IL-23p19 and p40 subunit mRNA in psoriasis vulgaris

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E Lee, I Cardinale, MF Chamian, WL Trepicchio, JL Oestreicher, D Pittman and JG Krueger. Laboratory for Investig. Dermatology, The Rockefeller University, New York, NY; Dept of Molecular Medicine, Genetics Institute, Cambridge, MA Psoriasis, the most prevalent T-cell mediated disorder affecting the general US population, is best characterized as a Type I immune deviation with increased expression of cytokines induced by interferon-γ (IFN-γ). Interleukin-23 (IL-23), a recently described hetreodimeric cytokine, has activity and structural similarity to IL-12, which is a pro-inflammatory cytokine. It shares the p40 subunit with IL-12 but the other subunit, named p19, is distinct from p35 of IL-12. We have examined the gene expression patterns of the IL-23 p19 subunit by quantitative RT-PCR and report an increase in relative gene expression in lesional skin compared to non-lesional skin (22.3-fold; p=0.001). The p40 subunit, shared by IL-12 and IL-23, also increases compared to non-lesional skin (11.6-fold; p=0.003), while the p35 subunit does not demonstrate any significant change. Knowing that the p40 subunit is shared between IL-12 and -23 creates a more complicated signaling pathway. While the function of IL-23 in psoriatic skin as an inflammatory mediator is unknown, this data supports a role for this new cytokine in the inflammatory environment.

0676

MMLV vectors efficiently transduce collagen VII cDNA and allow preclinical gene therapy trials in dystrophic EB

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Gene therapy of genodermatoses is limited by the availability of vectors able to accommodate and transfer large cDNAs. Since MoMLV retroviruses are the most used vectors for gene therapy, we assessed their suitability to correct the genetic defect of keratinocytes of patients with recessive dystrophic epidermolysis bullosa (RDEB). RDEB is an inherited skin blistering disorder due to mutations in collagen VII which assembles into the anchoring fibrils of the papillary dermis. The collagen VII cDNA (9 kb) cloned into a pLZRS retrovirus was transduced into primary collagen VII-null RDEB keratinocytes and fibroblasts. The highly efficient gene transfer resulted in secretion of the recombinant polypeptide that displayed the structural properties of the wild-type collagen VII. The transduced keratinocytes lost the hypermotility characteristic of the RDEB cells, and efficiently deposited immunoreactive collagen VII at the dermal-epidermal junction of transplantable ex-vivo reconstructed skin. The artificial skin grafted onto SCID mice generated a basement membrane with mature hemidesmosomes and anchoring fibrils. This approach of genetic therapy was validated in a spontaneous animal model of RDEB dog in which mutated collagen VII molecules are expresssed and assembled in abnormal anchoring fibrils. Ex vivo transduction of the dog RDEB keratinocytes with MoMLV retroviruses expressing the dog wild-type collagen VII cDNA and construction of artificial skin using the infected cells demonstrated the stable reversion of the RDEB phenotype. Our results demonstrate that the currently utilized retroviral vectors efficiently transfer the large collagen VII cDNA into primary RDEB keratinocytes and produce transplantable epithelia permanently expressing the curative transgene. They also open new perspectives for the use of retroviral vectors in the transfer of genes encoding large polypeptides.

0677

Genetic heterogeneity of cutis laxa: a heterozygous tandem duplication within the fibulin-5 gene

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Inherited cutis laxa is a connective tissue disorder characterized by loose skin with variable internal organ involvement, resulting from paucity of elastic fibers. Frameshift mutations in the elastin gene have previously been reported in three families with autosomal dominant inheritance, and a family with autosomal recessive cutis laxa was recently reported to have a homozygous missense mutation in the fibulin-5 gene. In this study, we analyzed the gene expression of elastin and fibulins -1 through -5 in fibroblasts from five cutis laxa patients. One patient was found to express both normal (2.2 kb) and mutant (2.7 kb) fibulin-5 mRNA transcripts. The larger transcript contains an internal duplication of 483 nucleotides, resulting in the synthesis and secretion of a mutant fibulin-5 protein with four additional tandem calcium-binding epidermal growth factor-like motifs. The mutation arose from a 22 kb tandem gene duplication, encompassing the sequence from intron 4 to exon 9. No fibulin-5 or elastin mutations were detected in the other patients. The results demonstrate that a heterozygous mutation in fibulin-5 can cause cutis laxa, and also suggest that fibulin-5 and elastin gene mutations are not the exclusive cause of the disease.

0678

Differential regulation of the human hairless promoter

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Mutations in the human hairless gene (hr), which is expressed predominantly in the skin and brain, are responsible for atrichia with papular lesions. Expression of hr in the skin is regulated in a hair cycle-dependent manner. During catagen, hr expression is differentially regulated among the different cellular compartments of the hair follicle (HF) itself, and abrogation of hr leads to the loss of structural integrity of the HF in humans and mice. Although the tightly regulated control of hr expression is essential for HF cycling, the elements which regulate expression remain unknown. In order to further understand the control of hr expression, we previously cloned the human hr promoter and identified a thyroid hormone response element (TRE) at 2.7 kb upstream of the CAP site. hr has been identified as an early response gene to thyroid hormone (T3) stimulation in the developing rat brain, however, whether the same regulation occured in skin remained undetermined. We previously demonstrated T3 transcriptional upregulation through the hr TRE in neuroblastoma cells but not in keratinocyte cell lines, suggesting that the regulation of hr in skin is TR-independent. Through transient transfection of additional luciferase gene reporter constructs linked to a series of further delimited promoter deletions, the signal sequences responsible for the high basal level of expression in keratinocytes has been narrowed, and a unique sequence responsible for the differential activity of the promoter identified. Furthermore, the activity of the sequences in contexts other than the hr promoter was examined by the addition of regulatory sequences to a minimal promoter in reporter gene assays. Further dissection of cis-acting regulatory regions of the human hr promoter will provide new insight into the mechanisms of HF cycling and the highly controlled expression of hair cycle genes.

0679

Different banal nevi have different gene expression patterns

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Gene expression patterns in common nevi may reflect the earliest genetic changes leading to melanoma. We have cultured common compound nevi from adults as well as a giant congenital nevus from a child, and normal skin melanocytes, and compared their gene expression profiles. Our hypothesis was that we would see a single major pattern of gene regulatory differences between all banal nevi and normal melanocytes from the same person cultured under the same conditions. Melanocytes and nevomelanocytes were grown in specialized media supplemented with specific growth factors. All were harvested at the 3rd or 4th passage. RNA was extracted using a Qiagen kit, and Poly-A + RNA was amplified using the Arcturus system. cDNA was reverse transcribed incorporating the Cy3 fluor and this sample cDNA was applied in to a 6912 spot DNA-microarray simultaneously with similarly amplified Cy5 labeled reference cDNA (Stratagene). Pattern detection was sought using K-means clustering and principal component analysis with Spotfire. Comparisons were made between all normal skin melanocytes, between each patient's melanocytes and their nevi, as well as across all samples. Curiously, one large group of genes had a decrease in expression in normal melanocytes correlating with the age of the patient from which the melanocyte cultures were started. Multiple cultures from one patient showed only slight differences between each other and that same patient's normal skin. In other cases, nevus cultures from the same patient demonstrated different patterns of gene expression, in one case having a similarity to the congenital melanocytic nevus culture. We conclude that different nevi even from the same person can express remarkably different patterns of gene expression, even though all nevi samples in these experiments were banal compound nevi derived from persons thought to be at a normal melanoma risk. In no case was a known growth regulatory pathway clearly evident. We feel this technique will be useful, as much larger numbers of nevi of different histological subtypes and from patients at risk for melanoma are sampled.

0680

Interaction of hairless and TR is dispensable for hair cycling

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Atrichia with papular lesions (APL, MIM 209500), is a rare autosomal recessive disease characterized by early-onset of atrichia, followed by a papular eruption within the first years of life. Recent studies demonstrating linkage to chromosome 8p21 and further mutation detection in the hairless gene (HR) have established the molecular cause of APL. In this study, we describe a 16 year old female with APL, and identified a homozygous missense mutation in hr, designated D1012N in the hr-thyroid receptor interacting domain 2 (TRID2). Recently, in vitro interaction assays identified two clusters of hydrophobic residues within hr, at positions 790 to 804 and 1001 to 1015, named TRID1 and TRID2 respectively. Interestingly, these TRID motifs are similar to the ones reported in the nuclear receptor corepressors N-CoR and SMRT which form multiprotein complexes that often include histone deacetylase. To determine whether this mutation abolishes the hr-TR interaction mediated by TRID2, we performed transient transfection with plasmids encoding the mutant D1012N hr and TR, followed by co-immunoprecipitation. We found no disturbance in the interaction or localization, and have therefore determined that this mutation does not dramatically affect the integrity of the hr-TR interaction, nor does it alter the nuclear distribution of the two proteins. This result suggests that the TRID2 domain is dispensable for the hr-TR interaction in the skin. The mutation D1012N must therefore be involved in disruption of hr protein folding and protein-protein interaction, or with other component(s) of the hairless regulatory complex that remain to be identified.

0681

Genetic heterogeneity in the multiple lentigines/LEOPARD/Noonan syndromes

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Multiple Lentigines syndrome (MLS) is a rare autosomal dominant disorder, characterized by diffuse cutaneous lentigines that may occur with non-cutaneous manifestations. Recent reports characterize the Multiple lentigines (MLS)/LEOPARD syndrome (MIM 151100) and the Noonan syndrome (MIM 163950) as allelic disorders, based on the finding that both syndromes may be caused by mutations in the PTPN11 gene (MIM 176876). We previously excluded PTPN11 as the cause of MLS based on genetic linkage studies in a large family with autosomal dominant inheritance of the disorder suggesting that the MLS is a distinct entity and is not a subtype of the Noonan Syndrome. As part of that study, we also excluded the genes responsible for other clinically overlapping disorders, including von Recklinghausen neurofibromatosis (NF1), Carney syndrome types I and II (CNC1 and CNC2), Peutz-Jeghers syndrome (PJS), and Cowden disease (CD). We show that MLS in this family is due to a mutation in a novel gene that maps to the long arm of chromosome 6(q24-25). The highest LOD seen in the initial genome screen was 2.29 for marker D6S1581, which maps to the long arm of chromosome 6. Linkage informativeness for this region was increased by genotyping markers from a higher density (5 cM) mapping set. The results are definitive in positioning the MLS locus in the 10-cM region between markers D6S308 and D6S411 based on a maximum LOD of 4.129 at marker D6S1654, (odds favoring linkage > 10,000:1) and a 1-lod confidence interval. The segregation of chromosome 6q haplotypes supports the statistical evidence for linkage with 3 recombinants confirming a position for the MLS locus proximal to marker D6S441 and one recombinant confirming a position distal to marker D6S308. The 12-13 Mb region between D6S308 and D6S411 contains nearly 100 known or predicted genes. Candidate genes are being studied to identify the responsible gene.

0682

Mutations in fumarate hydratase cause hereditary leiomyomatosis and renal cell carcinoma in North American families

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Hereditary leiomyomatosis and renal cell cancer (HLRCC) is a genodermatosis characterized by smooth muscle tumors of the skin and uterus and/or renal neoplasms. Although the identification of germline mutations in fumarate hydratase(FH) supports FH as the susceptibility gene for HLRCC, its role in North American families is unknown. Using DNA sequence analysis we screened for germline mutations in FH in 35 North American families with cutaneous leiomyomas. We also investigated the health problems associated with HLRCC particularly uterine leiomyomas (fibroids) and renal tumors. Sequence analysis revealed germline mutations in FH in 31/35 families. We found 20 different mutations in FH, of which 18 were novel. Of these 20 mutations, 2 were insertions and 5 were deletions that caused frameshifts leading to premature truncation of the protein, and 13 were missense mutations. Eleven unrelated families shared the R190H mutation and 2 unrelated families had the Y422C mutation. Eighty-one individuals (47 women and 34 men) had cutaneous leiomyomas. Ninety-eight percent (46/47) of women with cutaneous leiomyomas had uterine fibroids. Eighty-nine percent (41/46) of women with leiomyomas and uterine fibroids had a total hysterectomy, 44% of whom had a hysterectomy by the age of 30. We identified 14 individuals from 5 families with unilateral and solitary renal tumors. Six individuals from 4 families had papillary type II renal cell carcinoma and one individual from one family had collecting duct renal cell carcinoma. Renal cell carcinoma was associated with an aggressive clinical behavior. with 9/14 dead of metastatic disease within 5 years of diagnosis. Mutations in FH are associated with HLRCC in North American families. HLRCC is associated with clinically significant uterine fibroids and aggressive renal tumors. Our study expands the histologic spectrum of renal tumors and mutations in FH associated with HLRCC.

0683

Birt-Hogg-Dubé syndrome: identification of a novel gene and its clinical manifestations

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The Birt-Hogg-Dubé syndrome (BHD), a genodermatosis characterized by benign tumors fibrofolliculomas, renal tumors and spontaneous pneumothorax. Previously we localized the BHD gene to chromosome 17p11.2 by linkage analysis. Recombination mapping in BHD families delineated the susceptibility locus to 700 kb on chromosome 17p11.2. Sequence analysis of the 14 coding exons revealed frameshift or termination mutations in a panel of BHD families. Mutations were located along the entire length of the coding region excluding the 5'UTR. The majority of BHD mutations were predicted to truncate the BHD protein, folliculin, with a 44% frequency of insertion/deletion mutations within a hypermutable C_8 tract in exon 11. Tissue expression of the 3.8 kb transcript was widespread including kidney, lung, and skin. Folliculin, is a novel protein that was highly conserved across species. Genotype and phenotype features will be discussed in detail. BHD is the first gene associated with renal oncocytoma, chromophobe renal cancer, spontaneous pneumothorax and fibrofolliculomas.

0684

Study of the neuroprotective and anti-apoptotic properties of ETRY50, a new neurotrophic agent

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In vitro experiments carried out on the rat pheochromocytoma (PC12) cell line, considered as a model of peripheral neurons, show the properties of a new neurotrophic agent, capable to oppose to neuronal degeneration and death induced by oxidative stress and aging. In a first body of experiments NGF-differentiated PC12 cells were stressed by UVB-radiations or growth factor deprivation, and the protective effect of ETRY50 was assessed both qualitatively (microscopical monitoring of cell morphology) and quantitatively (dosage of LDH activity in the culture medium). The antioxidant properties of ETRY50 were determined with chemical tests, and its ability to induce neuronal differentiation as a NGF-like factor, was determined on undifferentiated PC12 cultivated in absence or with sub-optimal amounts of NGF. Finally, interleukines (Il-6, Il-10) and neuropeptides (NPY, VIP) production, and the expression of early (bax/Bcl2) and late markers (caspase 3) of the apoptotic process were measured by ELISA or RT- PCR. Sub-millimolar concentrations of ETRY50 provide effective protection against UVB irradiation and growth factor deprivation. Experimental data support the idea that the neuroprotective and anti-apoptotic properties of ETRY50 result from its strong free radical scavenging effect, together with its neurotrophic effect, promoting neuronal differentiation and life span prolongation. Prevention of apoptosis correlated well with retained neuron-like morphology (improved neurite outgrowth), and the neurochemical study provides some insights into ETRY50's mechanism of action (IL-6 overexpresion). This low molecular weight lipophilic compound appears as a promising agent capable to preserve the neurocutaneous network, bringing benefits in many physiopathological mechanisms involving cutaneous innervation, including age-related sensitivity loss and impaired immune function, pigmentation disorders or dry skin.

0685

Rapid measurement of serum cytokines in psoriasis patients and correlation with disease severity

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Psoriatic plaques have been shown to contain increased levels proinflammatory cytokines. In addition, serum levels of IL-6, IL-7, IL-8, and INF-γ have been reported to be significantly elevated in psoriatic patients with active disease. The objectives of this study were to evaluate serum cytokine profiles in psoriasis patients by a rapid new technique and to correlate these levels with disease severity. We analyzed single serum samples from 10 patients with active untreated psoriasis and 3 healthy volunteers for all the major type 1 and type 2 cytokines using the LINCOplex ELISA multi-analyte detection system. Disease severity, including erythema, induration, scale, and surface area, was assessed by the PASI. The Spearman rank order test was used to determine correlations between cytokine levels and disease severity indices. When compared to normal sera, IFN-γ was markedly elevated in all sera from psoriasis patients (mean ± Standard Error of Mean (SEM)=33.8 ± 1.3 for psoriatics versus mean ± SEM = 7.6 ± 2.2 for normals, P = 0.001) and positively correlated with all indices of disease severity (Spearman r = >0.6). IL-8 was moderately increased (mean ± SEM = 24.4 ± 1.8 for psoriatics versus mean ± SEM = 6 ± 2.4 for normals, P = 0.02) and positively correlated with the degree of erythema (Spearman r = >0.6). Mean IL-2 and IL-12 levels were not significantly elevated in sera from psoriasis patients. Interestingly, serum IL-10 levels were below detection levels in psoriatics compared to normals (mean ± SEM = 8.6 ± 2.7). In summary, a new ELISA system allowed rapid and reliable detection of numerous cytokines in single serum samples from patients with psoriasis; type 1 and pro-inflammatory cytokines were elevated in psoriatics and correlated with parameters of disease severity. We suggest this new tool will be useful in further understanding psoriasis pathogenesis and may allow for improved prognosis of individual patients.

0686

Epidermal growth factor activates calpain in keratinocytes via the MAP kinase signaling pathway and induces apoptosis

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Calpain, a calcium-activated cysteine protease in cytoplasm, plays a role in keratinocyte differentiation by contributing to profilaggrin processing. Calpain has two ubiquitously expressed isozymes, mu-and m-calpain. The signal transduction of epidermal growth factor (EGF) to Ras is involved in cell proliferation, differentiation, apoptosis and cell survival, depending on its signal strength and duration via Raf, PI3K and RalGDS. The Raf-MEK-ERK pathway is well characterized and induces several transcription factors. In this study, HaCaT keratinocytes were cultured with EGF with/without MEK inhibitor (PD98059), ERK2 inhibitor (5-iodotubercidin), PI3 kinase inhibitor (LY294002) and calpain inhibitor I. Subsequently, the processing of alpha-spectrin, one of the major calpain substrates, was observed using immunoblots. As a result, EGF alone induced the processing of alpha-spectrin, which was inhibited by PD98059 and calpain inhibitor I. However 5-iodotubercidin and LY294002 failed to inhibit this process. Furthermore, degradation of Bcl-2 was observed when HaCaT keratinocytes were cultured with EGF. This degradation was inhibited by calpain inhibitor I. Immunoblots revealed the early degradation of m-calpain molecule. These findings suggest that MEK induces m-calpain activation in HaCaT keratinocytes, which in turn triggers apoptosis via degradation of Bcl-2 molecules.

0687

MAPK pathway inhibition is correlated with the down-regulation of MMP-9 secretion induced by TNF-alpha in human keratinocytes

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MMP-9 is the major gelatinase able to degrade collagen IV secreted by keratinocytes actively involved in tissue regeneration, wound-healing or tumorigenesis. MMP-9 being the predominant protease secreted in vivo by activated human keratinocytes, the regulation of its secretion was investigated. Our previous studies had demonstrated a high migration and secretion of MMP-2 and -9 by ras-transfected HaCaT cells. Presently, our studies using zymography, ELISA and RT-PCR demonstrated that pro-MMP-9 (92 kDa) expression and secretion is up-regulated by TNF-alpha, a major pro-inflammatory cytokines involved in several pathologies. Under our experimental conditions, MMP-9 was essentially up-regulated via MAPK activation from Ras, Raf, MEK, Erk and AP-1 cascade, and NF-kappaB to a lesser extent whereas stress kinases, p38 MAPK and JNK were not implicated. The pretreatment of cells with specific inhibitors of MAPK pathway downregulated MMP-9 mRNA and protein expression. As from in vitro studies MMP-9 is only present on its inactive form, the regulation of its active form (86 kDa) induced by plasminogen is under investigation. MMP-9 activity being controlled by TIMP-1, collagenases as MMP-1 and -13, and stromelysin-1 (MMP-3), the activation of these enzymes by TNF-alpha was also verified. These data suggest that the control of MAPK transduction signal pathways may offer a novel therapeutic approach for specifically inhibiting MMP activities up-regulated in inflammatory disorders and cancerogenesis processes.

0688

Apoptosis and mitotic catastrophe in human keratinocytes expressing the protein kinase C delta catalytic domain

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Cell death by apoptosis is a general response of tumor cells to radiation or genotoxic drugs, however alternative cellular mechanisms, such as mitotic catastrophe, can also limit the lifespan of genetically damaged cells. In a wide variety of cells undergoing apoptosis, protein kinase C delta (PKCδ) becomes cleaved by caspase-3 in its hinge domain to generate a constitutively active catalytic domain fragment. PKCδ is the only PKC isoform proteolytically activated in human keratinocytes undergoing UV-induced apoptosis, and PKC activation is a required component of the UV apoptotic program. To elucidate the cellular effects of PKCδ cleavage in apoptosis, we generated a retrovirus encoding the catalytic domain of PKCδ (PKCδ-cat) corresponding to the fragment generated by caspase-3 cleavage. HaCaT keratinocytes infected with the PKCδ-cat virus were TUNEL positive and showed morphological and ultrastructural features of apoptosis. Analysis of DNA content by propidium iodide staining however identified a substantial cell cycle arrest in the G2/M phase and polyploidy, but not sub-G1 DNA content. The polyploidy was confirmed by morphological identification of interphase cells with multiple nuclei, a hallmark of mitotic catastrophe. Rhodamine 123 staining of PKCδ-cat-infected HaCaT cells also showed significant depolarization of mitochondrial membrane potential, which could be blocked by over-expression of Bcl-2 or Bcl-x_L. The inhibition of apoptotic cell death by Bcl-2 and Bcl-x_L further increased the accumulation of polyploid cells, indicating that apoptosis is an alternative fate or subsequent event to mitotic catastrophe. Expression of a PKCδ-cat ATP binding mutant (K378A) was not able to induce any of the observed changes, demonstrating that kinase activity of PKCδ-cat is required. These results indicate that the common apoptotic effector PKCδ-cat is also able to restrict cell growth by inducing mitotic catastrophe, and thus is an important target for killing tumor cells that are resistant to apoptosis.

0689

Quantitative analyses of mRNA expression for every member of the fibroblast growth factor family in skin at various stages of hair cycle

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The results of previous gene knockout experiments demonstrated the important role of fibroblast growth factor-5 (fgf-5) gene products in mouse hair cycle regulation. Our studies showed that the gene translates into two proteins (FGF-5 and FGF-5S) through generation of alternatively spliced mRNAs, and suggest that they have opposite effects on hair growth. FGF-5S expressed in hair follicles antagonized FGF-5's ability to suppress/shorten the anagen phase of the hair cycle. In addition, in vitro experiments suggested that the activities of FGF-5 and FGF-5S were exerted via specific receptor(s) on dermal papilla cells through inhibition of the release of an uncharacterized, growth-stimulating factor(s) from outer root sheath cells. Against that background, we have comprehensively profiled the dynamic expression of FGF in skin and hair follicles. This entailed quantifying the absolute copy numbers of the expressed mRNAs for virtually the entire FGF family (currently 22 members) in mouse skin at various stages of the hair cycle. We found that some of the members were highly expressed in the skin and/or hair follicles, and that the expression levels correlating uniquely with the hair growth cycle.

0690

Cyclic adenosine 3′,5′-monophosphate-elevating agents inhibit transforming growth factor-β-induced SMAD3/4-dependent transcription via a protein kinase A-dependent mechanism

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SMAD proteins transduce signals from the TGF-β receptors to regulate the transcription of specific target genes. In this report, we have investigated the effects of cyclic adenosine 3′,5′-monophosphate (cAMP), a key second messenger in the cellular response to various hormones, on SMAD-dependent signaling in human HaCaT keratinocytes. Using either an artificial SMAD3/4-dependent reporter construct or the natural TGF-β target, plasminogen activator inhibitor 1 (PAI-1), we show that membrane-permeable dibutyryl cAMP, and other intracellular cAMP-elevating agents such as the phosphodiesterase inhibitor isobutyl-methylxanthine, the adenylate cyclase activator forskolin, or exogenous prostaglandin E2 (PGE2), interfere with TGF-β-induced SMAD-specific gene transactivation. Inhibition of protein kinase A (PKA), the main downstream effector of cAMP, with H-89, suppressed cAMP-dependent repression of SMAD-driven gene expression. Inversely, co-expression of either an active PKA catalytic subunit or that of the CRE-binding protein (CREB) blocked SMAD-driven gene transactivation. cAMP-elevating agents failed to inhibit nuclear translocation and DNA binding of SMAD3/4 complexes but abolished the interactions of SMAD3 with the transcription co-activators CBP and p300 in a PKA-dependent manner. These results suggest that suppression of TGF-β/SMAD signaling and resulting PAI-1 gene transactivation by cAMP-inducing agents occurs via PKA-dependent, CREB-mediated, disruption of SMAD-CBP/p300 complexes.

0691

Comparison of site-matched papillary and reticular dermal fibroblasts

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Interfollicular skin contains two subpopulations of fibroblasts, papillary cells that reside in the superficial dermis adjacent to the epidermis and reticular cells that reside in the deep dermis. This study compares interactions between these two site-matched populations of fibroblasts with adult keratinocytes. Site-matched papillary and reticular cells produced significantly different amounts of signaling molecules that potentially affect keratinocyte proliferation and/or differentiation: keratinocyte growth factor (KGF), granulocyte-macrophage growth factor (GM-CSF), and interleukin-6 (IL-6). Papillary cells produced proportionally less KGF and more GM-CSF than did their reticular counterparts. Unstimulated papillary cells also produced less IL-6. Interactions between these two populations of fibroblasts and adult keratinocytes were also compared in an in vitro model where keratinocytes and fibroblasts were randomly mixed in a type I collagen matrix. Fibroblasts and keratinocytes spontaneously sorted with keratinocytes forming solid, but highly organized cellular masses that contained epidermal cells with varying degrees of differentiation. The appearance of the terminal differentiation marker filaggrin was more prominent when papillary cells were present. Furthermore, basement membrane formation was enhanced when papillary cells were present. These studies indicate that site-matched papillary and reticular dermal fibroblasts interacted differently with adult keratinoctyes. These results suggest that papillary fibroblasts are programmed to more effectively support the epidermis than are corresponding reticular cells. These results have significant implications for the production of skin equivalents and for enhancing our understanding of dermal fibroblast physiology.

0692

Evaluation of the effects of a zinc/iron solution on the migration of fibroblasts in an *in vitro* incisional wound healing model

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We have previously reported that in-vivo application of a new zinc/iron solution enhanced healing in acute partial thickness and second-degree burn wounds. There is evidence that zinc can enhance collagen synthesis but less is known about the role of iron. The purpose of this study was to determine which component(s) of the zinc/iron solution may be responsible for the enhancement of healing. Fibroblast cells in an in-vitro incisional healing model were grown to confluency on the bottoms of polystyrene well clusters. Digital photographs were taken with an inverted microscope at time zero after wounding. Subsequently, the wells were divided into eight treatment groups with two wells per solution. The treatment groups were as follows: growth media alone, distilled water, zinc, iron and zinc/iron solution. Growth media was added to all treatments except distilled water. After 24 hours of incubation, digital photographs were taken of the wound area with an inverted microscope. The percent migration of fibroblasts from baseline to 24 hours was measured using photographic digital planimetry. Results suggested that the combination of both zinc/iron solution was the most effective treatment when compared to zinc or iron alone. These results suggest a synergistic mechanism of action for the zinc/iron solution.

0693

Alteration of TGF-β/Smad pathway in aged and photoaged human skin

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In an effort to characterize TGF-β signaling and to determine its association with the aging and photoaging processes, we directly compared the expressions of TGF-β/Smad in intrinsically aged and photoaged human skin in vivo. This study examined expressions of TGF-β1, TGF-β2, TGF-β3, Smad 2, Smad 3, Smad 4, Smad 7, phosphated Smad 2 (pSmad 2), together with TβRI and TβRII, in tissue pairs of sun-exposed and sun-protected skin of both young (20~25y) and old (>75y) subjects by RNase protection assay and immunohistochemistry. The expression levels of TβRII mRNA and protein in the epidermis of forearm skin of old person were significantly lower than those of buttock and upper-inner arm skin of the same individuals. Immunohistology of TGF-β1 revealed prominent staining in the epidermis in buttock skin of young volunteers. This staining was substantially reduced in sun-exposed and sun-protected skin of old volunteers. In the epidermis, the expressions of Smad 7 mRNA in both the intrinsically aged and photoaged skin of old subjects were significantly higher than in the sun-protected skin of young subjects, and increased more in photoaged epidermis. The level of Smad 3 among Smads was expressed at greater level in the dermis of photoaged skin when compared with sun-protected skin of young and old persons. A decreased immunoreactivity for pSmad 2 in the epidermis of forearm skin was seen versus intrinsically aged buttock skin. This decrease was also found in the epidermis of upper-inner arm skin from old individuals compared with young individuals. These results suggest that UV-induced down-regulation of TβRII and concerted over-expression of Smad 7 may trigger an inhibition of TGF-β-induced phosphorylation of Smad 2. This impaired TGF-β/Smad signaling could lead to decreased collagen synthesis and wrinkles observed especially in photoaged skin.

0694

Smads regulate collagen gel contraction by human dermal fibroblasts

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Transforming growth factor-beta(TGF-beta) is a cytokine that enhances wound healing, a complex process encompassing a number of overlapping phases including wound contraction. The Smad family of signaling proteins functions as a principal transduction expressway from the TGF-beta receptors. However, their roles in wound contraction remain unclear. Here we examined the effect of Smad proteins on wound contraction using an in vitro type I collagen gel contraction assay with human dermal fibroblasts infected with adenoviruses carrying Smads. Addition of TGF-beta1 to dermal fibroblasts embedded in collagen gel significantly enhanced their contraction. TGF-beta1-mediated collagen gel contraction was further enhanced by Smad3, a major signal transducer in the Smad family, in fibroblasts. Interestingly, overexpression of Smad3 alone significantly enhanced collagen gel contraction by fibroblasts when compared with fibroblasts overexpressing a control lacZ. In this condition, even a very low concentration of TGF-beta1 that did not affect the collagen gel contraction by itself enhanced the contraction by fibroblasts overexpressing Smad3, suggesting synergistic effect of TGF-beta1 and Smad3. In contrast, TGF-beta1-mediated collagen gel contraction was suppressed by overexpression of Smad7, a major inhibitory regulator in the Smad family, in fibroblasts. In addition, RT-PCR analysis showed that the differential effects of Smad3 and Smad7 appeared to be associated with TGF-beta1 mRNA levels induced in fibroblasts. Thus, modulation of Smad3 or Smad7 expression in dermal fibroblasts affected their contraction of collagen gels possibly by regulating both TGF-beta signaling and TGF-beta expression in fibroblasts.

0695

Expression and function of neurotrophins and neurotrophin receptors in human keratinocytes

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The family of human neurotrophins (NTs) includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. The aim of the present study was to evaluate the expression and function of NTs in human keratinocytes. RT-PCR and ELISA, respectively, revealed that human keratinocytes synthesize and release BDNF, NT-3 and NT-4/5. In particular, keratinocyte stem cells express and secrete higher levels of NGF as compared to "young" transit amplifying (TA) cells (pg/ml 851±40 vs. 618±70, p<0.05), while NGF levels are almost undetectable in TA cells. Keratinocytes express the low-affinity p75 receptor which binds all NTs, the high-affinity NGF receptor trkA and the NT-3 receptor trkC. By contrast, keratinocytes express only a truncated form of trkB, the BDNF high-affinity receptor, with an altered cytoplasmic domain. Furthermore, NT-3 significantly stimulates keratinocyte proliferation in a dose dependent manner, as shown by 3H thymidine incorporation (100 ng/ml, p<0.01). Moreover, addition of an anti-NT-3 neutralizing antibody blocks keratinocyte proliferation (1:5, p<0.05). On the contrary, no effect of NT-4/5 or BDNF on keratinocyte growth is observed. NTs other than NGF fail to protect keratinocytes from UVB-induced apoptosis (TUNEL). Interestingly, while NGF decreases upon UVB irradiation, NT-3 and NT-4 are up-regulated. UVA dose-dependently increases NT-3 levels, as compared to sham (pg 585±100 vs. 178±21, p<0,05), whereas it does not affect the other NTs. NT-3 and NGF stimulate each other release, while they fail to affect either BDNF or NT-4/5, as shown by ELISA. These results strongly suggest that a complex neurotrophin network exists in human keratinocytes. In this context, NGF and NT-3 seem to play an important role.

0696

High-density oligonucleotide array analysis of interleukin-6 sensitive and resistant human melanoma cells

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Cytokine resistance is a well described phenomenon of advanced melanoma and has been established for interferons and interleukin-6 (IL-6). The molecular base for IL-6 resistance in melanoma still remains elusive. We performed high-density oligonucleotide array analysis of two representative human melanoma cell lines, WM35 (derived from an early primary tumor; IL-6 sensitive) and WM9 (derived from a metastasis; IL-6 resistant) stimulated with IL-6 for various time points (1, 3, 6 and 12 h). Hybridization of biotinylated cRNA samples was performed on HuGeneFL Affymetrix gene chips. Data evaluation, cleansing, extraction and analysis of the approximately 5600 genes was performed by the Gene Data Analyst vs.1 Expressionist software. The filter criteria included a minimum threshold of 100, normalization by the logarithmic mean and a quality setting of p<0,04. Only robust changes with a change factor of >3 were regarded as significant. IL-6 treatment of WM35 cells resulted in a time-dependent induction of several known IL-6 responsive genes such as the transcription factors NF-IL6beta, junB and c-fos, none of which being regulated in WM9 cells. In contrast, IL-6 treatment of WM9 cells led to an upregulation of proleukin-1beta and the chemokine IL-8, the latter a known growth factor for melanoma. By applying the above criteria, several novel IL-6-regulated functional gene clusters were identified in WM35 cells which included transcription factors (e. g. transducin-like enhancer protein, hypoxia-inducible factor 1alpha), cell cycle players (e. g. cdc2-related kinase), signal transduction components (e. g. dual-specificity phosphatase-5/hVH3/B23) and enzymes crucially involved in DNA synthesis (e. g. deoxyguanosine kinase). None of these genes was significantly altered by IL-6 in WM9 cells. Further studies are required to confirm these data at the protein level and to assess the relevance of the identified gene products with regard to IL-6-induced growth arrest and IL-6 resistance.

0697

Dual mechanisms of (-)-epigallocatechin-3-gallate-induced cell survival in human epidermal keratinocytes

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Green tea is widely consumed as a beverage. Its potential beneficial effects such as anti-cancer and antioxidant properties may be mediated by (-)-epigallocatechin-3-gallate (EGCG), a major constituent polyphenol in brewed green tea. Recently, EGCG was found to have a pronounced growth inhibitory effect on cancer cells, but not on normal cells. In this study, the effects of EGCG on cell proliferation and UV-induced apoptosis was investigated in normal epidermal keratinocytes, and the molecular mechanisms through which EGCG may act in vivo and in vitro were determined. The topical treatment of EGCG to aged human skin stimulated the proliferation of epidermal keratinocytes, and resulted in an increase in the epidermal thickness. In addition, the topical application of EGCG to human skin was also observed to inhibit the UV-induced apoptosis of the epidermal keratinocytes. EGCG increased the phosphorylation at the 112th and 136th serines of the Bad protein in epidermal keratinocytes. EGCG-induced Erk phosphorylation was found to be critical for the phosphorylation of the 112th serine, and EGCG-induced activation of the Akt pathway is involved in the phosphorylation of the 136th serine, respectively. Furthermore, EGCG increased the Bcl-2 expression level, but decreased the Bax expression level in epidermal keratinocytes, leading to an increase in the Bcl-2 to Bax ratio. In conclusion, EGCG promotes keratinocyte survival, and inhibits the UV-induced apoptosis by dual mechanisms; by phosphorylating the 112th and 136th serines of the Bad protein through the Erk and Akt pathways, respectively, and by increasing the Bcl-2 to Bax ratio. The two proposed molecular mechanisms associated with the EGCG-induced cell proliferation may act under different kinetics to promote the survival of keratinocytes.

0698

Keloid-derived fibroblasts show "myofibroblast" phenotype associated with IFN-gamma resistance

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Interferon-gamma (IFN-gamma) has been noted as a potential therapeutic agent for various fibrotic disorders, in part, through its antagonistic effect on transforming growth factor-beta (TGF-beta). Keloid is a fibrotic skin disorder that results in an excessive deposition of extracellular matrix, which is associated with altered-expression of or -responses to TGF-beta in dermal fibroblasts. We sought to determine whether IFN-gamma antagonized TGF-beta-mediated responses observed in keloid-derived dermal fibroblasts. Type 1 collagen production, fibroblast contractile activity, and alpha-smooth muscle actin (alpha-SMA) expression were assessed by using Western blotting, an in vitro type 1 collagen gel contraction assay, and immunofluorescence study in normal and keloid-derived human dermal fibroblasts in the presence or absence of IFN-gamma and/or TGF-beta. In contrast to normal dermal fibroblasts, IFN-gamma did not inhibit TGF-beta-induced type 1 collagen production, contractile activity, and alpha-SMA expression in keloid-derived dermal fibroblasts. Interestingly, keloid-derived dermal fibroblasts constitutively expressed type 1 collagen and alpha-SMA with increased capacity to contract a collagen matrix. In contrast to normal dermal fibroblasts, keloid-derived dermal fibroblasts showed "myofibroblast" characteristics that were associated with unresponsiveness to IFN-gamma. Thus, IFN-gamma may not be therapeutically useful for keloid and clarification of the mechanisms underlying the IFN-gamma resistance would be important for better understanding of the pathophysiology.

0699

Keratinocyte-specific HB-EGF-deficient mice show marked retardation of skin wound healing

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Cutaneous wound healing requires precise coordination of epithelization, dermal repair, and angiogenesis. For epithelization, the most important growth factor is the EGF family. Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family, stimulates the growth of normal human keratinocytes in an autocrine manner. HB-EGF is thought to play an important role in skin wound healing, although there have been no studies of the involvement of HB-EGF in skin wound healing in vivo using a mouse model. Since germline targeting of the HB-EGF gene resulted in embryonic lethality or severe growth retardation, we generated keratinocyte-specific HB-EGF-deficient mice (KS-HB-EGF-/-mice) using Cre/loxP technology in combination with the keratin 5 promoter. A cutaneous wound was made on the backs of mice using a 6-mm punch biopsy and wound closure was measured by migration of the epidermal edge stained with anti-keratin antibody. There was no difference in wound closure between KS-HB-EGF-/- and wild-type mice on day 3. However, wound closure was markedly retarded in KS-HB-EGF-/- mice compared to wild-type mice (30.7 and 44.5% on day 5, 78.3 and 96.7% on day 9 in KS-HB-EGF-/- and wild-type mice, respectively). To further clarify the role of HB-EGF in cutaneous wound healing, we performed a scraping wound assay using normal human keratinocytes. HB-EGF mRNA was rapidly induced, optimally 2.8 fold 2 h after scraping, although slight increases in TGF-alpha, amphiregulin, and epiregulin mRNA were observed. Furthermore, the addition of recombinant HB-EGF accelerated wound closure in the scraping wound assay, whereas anti-HB-EGF blocking antibody inhibited wound closure. Combined, HB-EGF plays an important role in skin wound healing by accelerating keratinocyte migration. In conclusion, we clearly demonstrated for the first time that HB-EGF is the most important growth factor in the epithelization of skin wound healing in vivo using keratinocyte-specific HB-EGF-/- mice.

0700

Distribution of HB-EGF and TGF-α in normal and psoriatic skin

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TGF-α and HB-EGF are members of the EGF family that regulate keratinocyte proliferation. Several reports have suggested differences in the regulatory system for their expression in the epidermis. We examined the distribution and dynamics of TGF-α and HB-EGF in normal and psoriatic epidermis, using immunohistochemical and in situ hybridization procedures. Immunohistochemical analyses showed that HB-EGF and TGF-α were distributed in the divided area of normal epidermis, with the former found in the basal layer and the latter in the suprabasal layers. With the increase of epidermal thickness from the peripheral area to the center of psoriatic plaque, a gradual increase of HB-EGF-expressing layers was observed from the near side of the basal layer. TGF-α expressing cells gradually decreased, accompanied by an increase of HB-EGF-expressing cells, and finally disappeared from the epidermis, whereas HB-EGF was produced throughout the whole area. In the center of psoriatic plaque areas that accompanied more seriously developed epidermal hyperplasia along with invagination of the epidermis, TGF-α expression was observed in the core part of the stratum papillare, whereas HB-EGF was expressed throughout the whole area of the epidermis. The results of in situ hybridization completely agreed with those of immunohistochemical studies for TGF-α. Furthermore, the expression pattern of K10 keratin, which is a differentiation marker for epidermal keratinocytes, resembled that of TGF-α, and in vitro analyses using cultured keratinocyte showed that keratinocytes differentiated by calcium treatment or stratification began to express TGF-α. We concluded that TGF-α expression is triggered in a differentiation-associated manner in keratinocytes and the number of HB-EGF expressing cells is closely correlated to keratinocyte proliferation in the epidermis.

0701

Deficient pro-IL 16 expression in stage III CTCL

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Pro-interleukin-16 has been identified as a novel 80 kDa regulatory protein of T cell growth. The presence of pro-IL-16 in the nucleus results in cell cycle arrest while the loss of nuclear pro-IL-16 following T cell activation facilitates cell cycle progression. In previous experiments we have observed that T cell lines derived from Sezary Syndrome (SS) patients, HUT78 and H9, either completely lack pro-IL-16 protein, or express a mutated form of pro-IL-16 that does not translocate into the nucleus, leading us to investigate the ability of primary CTCL T cells to generate pro-IL-16 protein. Lymphocytes were isolated from 5 patients diagnosed with stage III CTCL. Cell lysates were assessed by western blot analysis using a monoclonal anti-IL-16 antibody. We determined that two patients lacked complete IL-16 protein, confirmed by northern blots and southern blots; and 2 patients expressed a truncated form of IL-16 (68kDa) not found in normal T cells. Only one patient expressed pro-IL-16 at the appropriate weight, however at greatly reduced levels. Using flow cytometric analysis for intracellular pro-IL-16, 80% of normal T cells contained high levels of pro-IL-16. In contrast, cells from SS patients, where pro-IL-16 could be detected, had significantly lower expression, ranging from 1.5% to 31% by FACS analysis. We next correlated expression of pro-IL-16 with CD25 and found that there was a negative correlation, consistent with the concept that pro-IL-16 functions to limit cell cycle progression. Interestingly, all SS T cells appeared to express lower levels of pro-IL-16 as compared with T cells from normal individuals, suggesting that all T cells are altered in association with SS. These preliminary data indicate that T cells from SS patients either lack IL-16 protein completely or express significantly reduced pro-IL-16 levels. The overall reduced level of pro-IL-16 protein in SS cells likely represents a contributing factor to the dysregulated growth associated with CTCL.

0702

Lack of macrophage migration inhibitory factor significantly delays cutaneous wound healing

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Macrophage migration inhibitory factor (MIF) has emerged as a potent pro-inflammatory factor. MIF is induced by various stimuli such as wounding and infection and regulates inflammatory and immunological responses. We have previously demonstrated that increased MIF expression in the wound healing process in rats and MIF production by wounded fibroblasts was increased by the endotoxin (lipopolysaccharide, LPS). In order to evaluate the function of MIF, we studied the cutaneous wound healing process using an MIF knockout (KO) mice that we recently produced. We examined fifteen MIF KO and wild type (WT) mice in each group. Following the excision of a 4X4 mm biopsy wound from dosal skin of MIF KO mice and WT mice, healing was significantly delayed in MIF KO mice compared to WT mice ($p<0.001$). LPS treatment at concentrations of 1-2 ug/ml in the culture medium significantly stimulated cultured fibroblast proliferation in WT mice, compared to MIF KO mice fibroblasts after incubation for more than 5 days. Additionally, a significant increase in fibroblast migration was observed from WT mice compared to MIF KO mice. Therefore, excessive expression of MIF may directly or indirectly stimulate cell proliferation and migration, which profoundly modulates the wound healing process. These data clearly indicated that MIF may be crucial for cutaneous wound healing.

0703

Differential expression of angiogenic cytokines in atypical scleroderma induced by malignancies

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Pseudoscleroderma or atypical scleroderma as a paraneoplastic syndrome is a rare condition. We treated 5 patients considered as atypical scleroderma induced by malignancies, i.e. paraneoplastic syndrome from 1995 to 2001 in our department. This is a 7 % out of all referred patients of systemic sclerosis during the period. In all five cases, lung fibrosis or esophageal dysfunction was not detected. Antinuclear antibody was negative in 4 out of 5 cases. Raynaud's phenomenon was not observed. We detected malignancy (3 lung and 1 nasopharyngeal) during the intensive investigations. One patient revealed extensive skin sclerosis on hands and forearms after she developed metastatic beast cancer. Three cases which lung cancers were successfully removed. As angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and their receptors expression were investigated both in tumor and in the sclerodermatous skin lesion of each case by immunohistochemical method. We already reported serum bFGF or VEGF level was elevated in patients of systemic sclerosis (SSc). Serum bFGF level in 5 atypical cases was elevated in comaparison with normal controls or systemic SSc patients. Serum VEGF level in 5 atypical cases was elevated compaired with normal controls. However there was no significant difference between atypical cases and usual SSc patients. Histopathologically more prominent lymphocytic infiltration was observed in the sclerodermatous dermis of atypical cases. Strong expression of bFGF was observed not only in endothelial cells and keratinocytes but also in fibroblasts. The expression of flag and bek was also elevated in fibroblasts of atypical cases. There was no significant difference of VEGF between in atypical cases and SSc specimen. The expression of cytokines of 4 biopsied or resected tumors were also investigated. Strong expression of bFGF and VEGF was observed in each cases. It was suggested bFGF, angiogenic and fibrogenic cytokine, is involved in the fibrogenesis of such cases.

0704

Novel small molecule antagonists of interleukin 7 signal transduction

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Interleukin 7 (IL-7) is a growth factor for early B and T cells that protects cutaneous T-cell lymphoma (CTCL) cells from apoptotic cell death. IL-7 transgenic mice develop a cutaneous lymphoproliferative disorder and lymphomas. JAK3 tyrosine kinase is essential for signal transduction in response to IL-7. Therefore, inhibitors of JAK3 kinase block the biological effects of IL-7 and may have therapeutic value. A cell-based proliferation assay has been developed for high-throughput screening. By differential screening with IL-7 dependent (JAK3) and IL-3 dependent (JAK2) cell lines, we found two compounds with significantly increased potency and another compound that appears specific for JAK3. Lymph nodes of IL-7 transgenic mice are 5-10 times larger than those of normal mice and are readily visualized by MRI imaging. The efficacy of one compound, 5H4, was evaluated by treating 4 IL-7 transgenic mice with 5H4 by intraperitoneal injection for 16 days. Lymph nodes of individually tracked 5H4-treated mice reduced in size by 30% while those of vehicle-treated mice were unaffected. At necropsy the lymph nodes of the 5H4-treated mice appeared flaccid while the lymph nodes of the vehicle-treated mice were turgid. Histology of the 5H4-treated lymph nodes revealed loose porous tissue in contrast to the lymph nodes of vehicle treated mice, which were tightly packed. Small molecule kinase inhibitors block IL-7 signaling in vitro and in vivo and may have therapeutic value as pharmacological agents for the treatment of lymphomas and inflammatory disorders.

0705

Differential regulation of importin alpha 2 expression by TGFβ and IFN-γ in normal human epidermal keratinocytes

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One of the most important issues in the dermatological research is to clarify molecular mechanisms of signal transduction on the epidermal differentiation. Despite accumulation of numerous informations on this topics, almost no report on nuclear transportation regulation in the epidermal differentiation can be found. Here, we studied expression pattern of importin alpha (IMPA) 1, 2, 3, 4 and 5 subtypes, all of which are known to bind nuclear localizing signal (NLS) of various proteins, such as transcriptional factors, to regulate active nuclear transportation, in cultured normal human keratinocytes (NHK) and cultured normal human skin fibroblasts (NHF). Northern analysis exhibited that both NHK and NHF expressed all subtypes of IMPA. In the both cell types, IMPA2, 3 and 4 seemed to be major subtypes, while IMPA1 and 5 were minor in terms of relative mRNA expression. Next, we investigated modulation of IMPAs expression by TGF-β and IFN-γ, which are known to inhibit and induce epidermal differentiation, respectively. TGF-β stimulation of 5 ng/ml in NHK culture medium resulted in selective down-regulation of IMPA2 mRNA expression, and about 70% inhibition was observed after 24 hours. On the other hand, 100U/ml of IFN-γ in NHK culture specifically up-regulated IMPA2 expression. Maximal induction, which was about 2 times higher as compare to initial expression level, was observed after 6 hours incubation, and the expression level was gradually reduced to reach about 20% of initial expression level, suggesting dual regulation of IMPA2 by IFN-γ. Addition of cycloheximide, a protein synthesis inhibitor, antagonized the effect of both TGF-β and IFN-γ on IMPA2 expression, indicating requirement of on-going protein synthesis for IMPA2 regulation by TGF-β and IFN-γ. No other IMPAs were modulated by these cytokines. These data suggest that nuclear translocation mechanisms regulated by IMPA2 might be specifically involved in signal transduction pathway for the epidermal differentiation, at least induced by TGF-β and IFN-γ.

0706

Identifying a new role for the eicosanoid 12-HETE: modulator of keratinocyte migration

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Eicosanoids are widely recognized mediators of the inflammatory and immunologic responses, and their role as messengers in other cellular processes is rapidly expanding. We report here on the role of the eicosanoid, 12-hydroxyeicosatetraenoic acid (12-HETE), a product of arachidonic acid metabolism by the enzyme 12 lipoxygenase (LOX) in modulating keratinocyte migration. When confluent cultures of human neonatal keratinocytes are wounded, they generate 12-HETE, while unwounded cultures do not. Levels of 12-HETE vary over the course of wound healing, increasing about 30 fold over the 48 hours post-wounding. Inhibiting 12- HETE production in the wounded cultures by pretreating them with the LOX inhibitor nordihydroguaiaretic acid completely prevents cell migration and healing of the wound, which can be totally reversed by adding exogenous 12-HETE to the culture. Using LC/MS we identify 12-HETE in vivo, in the wound medium of suction induced blisters of human skin, further supporting the hypothesis that it modulates keratinocyte migration. Finally, its role in mediating migration can be derived by the ability of exogenously added 12-HETE to activate and phosphorylate the EGF receptor, and the downstream ERK1/2 MAP kinase, two required components of the pro-migratory signaling cascade in keratinocytes. Thus, we demonstrate that 12-HETE is generated during the course of wound healing and activates the EGF/ERK kinase pathway, culminating in keratinocyte migration. Exploiting this HETE-mediated signaling pathway may result in novel pharmacological approaches to enhance wound healing.

0707

Transcriptional profiling and dissection of signaling pathways responsive to proinflammatory cytokine TNFα

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The effects of TNFa are mediated by two well-characterized signaling pathways, NFkB and MAPK, which regulate expression of proinflammatory, immune response, proliferative and apoptotic genes. Using microarrays, we profiled the TNFa-mediated transcriptional changes in human epidermal keratinocytes during 48-hours post-treatment. TNFa induces proinflammatory and immunomodulatory genes, prominently chemokines, membrane receptors, MMPs and other proteases. Surprisingly, epidermal differentiation markers, basement membrane and antiapoptotic proteins are induced as well. Using parthenolide to block specifically the NFkB pathway, we found that this pathway provides the antiapoptotic stimulus. Microarrays of TNFa+Parthenolide-treated cells identify chemokines, epidermal differentiation markers and proteases among the NFkB-dependent genes. Microarrays predicted an important antiapoptotic role for CFLAR/FLIP in TNFa responses. Using RNA-interference to target CFLAR/FLIP or NFkB as control, and TUNEL microscopy, we confirmed the antiapoptotic functions of both CFLAR/FLIP and NFkB. Our data provide a global description of transcriptional effects of TNFa in keratinocytes, define the specific roles of the NFkB pathway and identify CFLAR/FLIP as an essential antiapoptotic protein.

0708

Basic fibroblast growth factor adminstration reduces scar formation in acute incisional wounds

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This study examines the relationship between the level of apoptosis and the degree of wound healing after the treatment of bFGF. Full-thickness incisional wounds were created on the backs of rats, and their healing processes were examined with respect to the level of apoptosis and the width of the granulation tissue or scar with/without the direct intradermal injection of recombinant bFGF in addition to suturing. The histology of the bFGF group on day 4 showed strong inflammatory reaction at the injured site when compared with the control. At the higher magnification of the bFGF-treated wound, infiltrated inflammatory cells, fibroblasts, and capillaries were evident. On day 7, there were increased accumulations in fibrotic tissue, cellular infiltration, and capillaries in the bFGF-treated wound when compared with the control. Morphometry of the wound tissue revealed that all parameters of the width in the group treated with 1 mcg of bFGF exceeded those in the control group on day 7. However, all parameters of the width on days 14 and 28 were significantly lower in thebFGF-treated group than in the control non-bFGF-treated group. The results of examinations of apoptosis by TUNEL metod and caspase-3 indicate that the exogenous administration of bFGF wound to acute incisional wounds accelerates apoptosis in the dermal healing tissue at the early phase, and that this apoptotic cell death contributes to the total reduction of cellularity at the late phase of wound healing and leads the scar less visible. Clinical significance of bFGF administration to reduce scar formastion was also evaluated and the results will be discussed.

0709

Flotillin-2 transformation of a non-metastatic melanoma line is associated with differential expression of PAR-1 and other important signal transduction genes

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Flotillin-2 (flot-2) is a 41.7 kDa, highly conserved caveolae/lipid raft associated protein. Metastatic melanoma cell lines and lesions have increased Flot-2 expression. When flot-2 was transfected into the non metastatic melanoma line (SB2), two separate transformed SB2 clones were highly tumorigenic and metastatic in nude mouse xenograft model. To determine how over-expression of flotillin-2 participates in progression of MM, we compared differential gene expression of transfected SB2 cells on two distinct oligonucleotide microarrays. When 100 ug of mRNA was hybridized to a cancer related DNA pathway array (CG-8) of 1100 unique sequenced 75-oligomers, only one gene, the thrombin receptor, PAR-1 was upregulated and was confirmed by Western blotting. Further experiments used either 5 ug of amplified mRNA or 120 ug of unamplified RNA with the standard microarray (CG4_1) of 2304 genes in duplicate. mRNAs from SB2-flot-2 transfected stable clones were compared to mRNAs from GFP-vector transfected lines. In the standard microarrays, unamplified samples revealed 34 upregulated and 12 down-regulated genes and amplified experiment showed 94 upregulated and 105 down-regulated genes (p= 0.05). Genes upregulated in flot-2 transfected lines include PAR-1 and uPA (urokinase type plasminogen activator), focal adhesion kinase (FAK) and PDZ-RhoGEF while RhoE, Stat-1, NFkBIA, Thrombospondin-1 and TIMP-2 were down- regulated. PAR-1 protein is known to transform melanoma and other candidate genes identified in this manner are also implicated in melanoma. This suggests that transformation of non-metastatic SB2 cells to metastatic melanoma cells may be mediated by genes controlling invasion, cell-cycle, angiogenesis, actin cytoskeleton organization, and apoptosis. Validation of differential gene expression identified by microarray analysis may increase our understanding of melanoma progression.

0710

Epidermal cell signalling and activation by a new diacylglycerol-rich extract

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Cell signalling and activation have been at the center of research for a long time. Among important cellular signalling paths is tyrosine kinase receptor activation that results in PKC phosphorylation and activation by diacylglycerol (DAG), which induces different important cellular responses. In order to induce direct and rapid cell activation, we developed a new DAG-rich extract and tested its effect and mechanisms of action on cultured human epidermal cells and fibroblasts, in dose course studies. PKC activation by the extract was investigated by immunoblotting studies that showed MARCKS substrate phosphorylation in cultured human cells through activated PKC. Moreover, the new extract increased basal cellular levels of the MAPK family (MAPK/ERK, p38MAPK and SAPK/JNK) and, interestingly, increased phosphorylated MAPK/ERK, which confirms MAPK/ERK activation after administration of the extract. This activation was dose-dependent. Since MAPK/ERK is implicated in different cell functions such as cell growth regulation and differentiation, additional studies on differentiation and proliferation markers were performed. These studies showed that PKC activation by the DAG-rich extract results in the activation of differentiation response more than proliferation. Interestingly, ATP evaluation studies revealed an increase in cellular level of ATP after administration of the extract. This increase started within 5 minutes, and reached its maximum after 15 minutes of administration. Furthermore, we monitored Ca2+ expression in selected single cells in response to administration of the extract, using a fluorescent probe and confocal microscopy. The selected cells elicited maximum fluorescence intensity within 60 s and dropped down to basal level thereafter. These ATP and Ca2+ results strongly suggest that DAG-rich extract activates cell targets other than PKC as well. These studies demonstrate that this new DAG-rich extract exhibits very interesting properties by combining different essential mechanisms of cell activation that are of interest for energizing and regenerating skin care products

0711

Cyclosporin A induces secretion of cyclophilin B in keratinocytes by a calcineurin-independent pathway

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Phorbol-ester induced cutaneous inflammation can be inhibited in SCID mice by cyclosporin A (CsA) indicating that CsA exerts pharmacological effects in skin, independent of T-cells. We have previously shown that in keratinocytes, CsA induces secretion of cyclophilin B (CypB), a CsA binding protein, via the constitutive secretory pathway. The aim of this study was to investigate the mechanism by which CsA induces CypB secretion. Firstly, the effects of calcineurin inhibition on CypB secretion were investigated. Keratinocytes were treated with the calcineurin inhibitors CsA, tacrolimus and cypermethrin; also CsH and rapamycin, an immunosuppressive drug that binds to FKBP12, but does not inhibit calcineurin. Secondly, as CsA inhibits keratinocyte growth, the effects of growth inhibition on CypB secretion were investigated. Keratinocytes were treated with TGF-β, retinoic acid and vitamin D3. Following incubation with the indicated agonists for 24h, 48h and 96h, conditioned keratinocyte culture medium was collected and analysed using Western blotting with a polyclonal antibody to CypB. As previously reported, CsA induced secretion of CypB. Neither the calcineurin inhibitors tacrolimus and cypermethrin, nor rapamycin or CsH induced secretion of CypB when compared with vehicle control at all timepoints. In addition, pre-treating keratinocytes with cypermethrin prior to adding CsA failed to inhibit secretion of CypB, indicating that inhibition of calcineurin is neither sufficient nor necessary for induction of CypB secretion. Secondly, TGF-β, retinoic acid and vitamin D3 failed to induce CypB secretion suggesting that the growth inhibitory effects of CsA are either completely distinct from other growth inhibitory pathways, or unrelated to CypB secretion. These findings provide evidence for the presence of a calcineurin-independent signalling pathway for CsA in keratinocytes and could account, at least in part, for the non-immune effects of CsA that are observed in skin.

0712

Activation of corticosteroid regulatory pathway during wound healing

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Corticosteroids are known as inhibitors of wound healing but the exact mechanism of this inhibition is largely unknown. Therefore, we used two different approaches, immunocytochemistry and global transcriptional analysis to identify the mechanism through which corticosteroids inhibit wound healing. Using immunocytochemistry, we discovered nuclear translocation of the glucocorticoid receptor (GR) at the wound edge that is completely absent from unwounded skin. This suggests that GR is released from its cytoplasmic inhibitor and translocated to the nucleus, directly indicating receptor activation during the process of wound healing. Using Affymetrix HU95A gene chips we compared the expression profiles of human skin 4, 48 and 96 hours upon wounding with human skin unwounded, but treated topically with corticosteroids. These analyses revealed that the expression pattern of corticosteroid treated skin does not have a significant overlap with the pattern of the 4 and 48 hours post-wounded skin. Importantly, we found a strikingly significant overlap with the 96 hours post-wounded skin: 33.2% genes identified as significantly regulated in corticosteroid-treated skin were also significantly regulated in skin 96 hours post wounding. Furthermore, we found 54% of those to be identically regulated, which means that 166 genes were identically regulated in both corticosteroid-treated skin and the skin 96 hours post-wounding. Specifically, we found a similar transcriptional pattern of genes that participate in adhesion, proliferation, migration, Wnt pathway and steroidogenic enzymes, to mention a few. Overall, we found that wound healing activates corticosteroid pathway whereas it dominantly inhibits EGF/TNFalpha pathway, resulting in inhibition of migration, increase of proliferation and change of adhesion of keratinocytes. We conclude that corticosteroid serve as natural inhibitors of normal wound healing, i.e. a switch that "resets" keratinocytes from their activated phenotype back to deifferentiating phenotype.

0713

New synthetic tripeptide with pleiotropic effects on epidermal cells

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One of the essential mechanisms of cell activation and signal transduction is the activation of cell membrane-related receptors, such as G-protein-coupled receptors. Being interested in the fundamental mechanisms of cell activation as an approach to cell aging, we developed a synthetic tripeptide, and tested its effects on cell activation. Cellular ATP assessment in human fibroblasts treated with 1% of the peptide, revealed an increase in cellular level of ATP. This increase started within 5 minutes, and reached its maximum after 15 minutes of administration of the peptide. Together with the peptide structure, this activation suggests that the peptide probably stimulates APnA (ATP reservoir molecules) receptors in the cells directly. As Ca2+ is important for cell signalling, and in order to investigate the effect of this peptide on cellular Ca2+ level, we monitored Ca2+ expression in selected single HaCaT cells in response to peptide administration, using a fluorescent probe and confocal microscopy. The selected cells elicited maximum fluorescence intensity after 90 s of peptide-treatment, and dropped down to basal level thereafter. Moreover, Erg expression, a very early marker of differentiation, was found to have increased in cultured epidermal HaCaT cells. This expression was studied, and it showed that the addition of the peptide into cultured cells stimulated cell differentiation. These results were confirmed by further studies on keratin synthesis. Additional tests on in vitro wound repair and protein synthesis confirmed general cell activation by this new peptide. These results strongly suggest that the structure of this new peptide, in addition to its great similarity to the described APnA receptor, is probably responsible for ATP induction. This peptide also shows evidence of cell activation via other G-protein-coupled receptors in epidermal cells. Taken together, these results demonstrate an interesting effect of the new tri-peptide on cell activation that is of great interest for topical skin regenerating and energizing products

0714

RelA-/- epidermis displays hyperproliferation with normal differentiation

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Overexpression studies that alter NF-kB function lead to dramatic changes in epidermal growth. It is therefore unexpected that no epidermis-intrinsic growth abnormalities are apparent in mice lacking the 4 of 5 NF-kB subunits examined to date. NF-kB subunits, however, display genetic redundancy and RelA/p65-deficient adult epidermis has not been studied due to embryonic lethality, leaving open a potential role for RelA in this setting. To address this, we generated developmentally mature RelA-/- skin. RelA-/- skin lacked RelA protein expression and was notable for marked hyperplasia, which remained stable over 8 weeks in vivo. RelA+/- control tissue was similar to wild-type RelA+/+ skin in every respect assessed. Hyperplasia in RelA-/- epidermis was due to increased cell division, as confirmed by >4-fold elevation in epidermal mitotic index. Unlike epidermis of Ikk1-/- mice or mice with epidermal IkBa overexpression, RelA-/- epidermis lacked alterations in cell death. These findings indicate that epidermal RelA deficiency leads to increased proliferation without altering apoptosis. RelA-/- epidermis also expresses correctly localized differentiation markers, indicating that RelA is dispensable for normal differentiation. Unlike Ikk2-/- tissue, RelA-/- skin lacked inflammatory cell infiltrate and induction of MCP-1, IL-b and TNFa. Moreover, keratinocytes were hyperproliferative in culture, indicating that increased cell division due to RelA loss is epidermal cell-autonomous rather than a reactive response to inflammation. Hyperproliferation is TNFR1-dependent because deletion of Tnfr1 normalized RelA-/- skin cell division and blocking antibodies to TNFR1 but not TNFR2 inhibited keratinocyte proliferation induced by NF-kB blockade. Furthermore, TNFR1-dependent JNK activation occurred in RelA-/- epidermis and JNK inhibition abolished IkBa mediated hyperproliferation. These findings suggest that NF-kB RelA antagonizes TNFR1-JNK epidermal proliferative signals and identify a non-redundant role for RelA in restraining epidermal growth.

0715

Antigen-specific regulatory function of anergic CD4$^+$ T cells is controlled by CTLA-4 and MAP kinase p38 signaling

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Previous results demonstrated that human IL-10-treated dendritic cells (DC) induce anergic CD4$^+$ T cells with antigen-specific regulatory function. This study analyzed altered cell cycle and signal transduction pathways in these anergic T cells compared to optimally stimulated T cells (cocultured with mature DC). Accumulation of the G_1-specific cyclin D3/D2-cdk4 complexes and high levels of the cdk inhibitor $p27^{Kip1}$ were observed in anergic T cells resulting in an inhibited activation of Rb and an arrest of cell cycle progression in the G_1 phase as compared to stimulated control T cells. Addition of IL-2 and functional blocking of the highly expressed CTLA-4 molecule on the anergic T cells induced a downregulation of $p27^{Kip1}$ and a complete inhibition of the antigen-specific regulatory function as demonstrated by high proliferation and enhanced IFN-γ production of cocultured T cells. Notably, analysis of signal transduction pathways demonstrated a markedly enhanced and sustained activity of the MAP kinase p38 during primary culture and after restimulation. Additional analysis of the kinase MAPKAP2/3, the downstream substrate of p38, revealed a significantly increased activity, suggesting an altered critical pathway of signal transduction in anergic T cells. Inhibition of the MAP kinase p38 using the specific inhibitor SD203580 completely impaired the induction of anergic CD4$^+$ T cells as demonstrated by restimulation experiments and IL-2 production. Importantly, compared to control T cells these T cells also lost their antigen-specific suppressor function. Our data show an important role of CTLA-4 and MAP kinase p38 signaling for the induction of antigen-specific anergy and regulatory function in CD4$^+$ T cells induced by IL-10-treated DC.

0716

Psoriasis-like phenotype induced by constitutively active Rac1 overexpression in the basal epidermis

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Rac1 GTPase promotes cell proliferation; however, its role in proliferative skin disorders remains unclear. To study the role of Rac1 in the skin, we cloned myc-tagged V12Rac1 cDNA into vectors with K14 or K1 promoters and produced transgenic mice overexpressing constitutively active Rac1 in the basal or suprabasal epidermis, respectively. Multiple transgene copies were detected in V12Rac1 +/- mice by Southern blot and Rac1 transgene expression was verified by Western blot. Between 1-3 weeks, K14V12Rac+/- mice showed generalized, indurated, erythematous, hyperkeratotic skin eruptions. Stratified squamous mucosal tissues were largely unaffected. Histology showed greatly thickened epidermis, parakeratosis, focal hypogranulosis, elongated rete ridges and dilated blood vessels in dermal papillae. There was an associated mixed inflammatory infiltrate with neutrophils, and immune cells were also occasionally noted in the epidermis, including stratum corneum. Microarray analysis of K14V12Rac1+/- skin showed overexpression of many genes that were also found to be overexpressed in psoriatic skin. After 3 weeks, the skin changes in K14V12Rac1+/- mice subsided, however pruritic, persistant, indurated, erythematous plaques of epidermal hyperplasia could be readily induced by minor trauma. Treatment with topical corticosteroids and occlusion improved the skin lesions. Interestingly K1V12Rac1+/- mice showed no skin changes, suggesting that overexpression of active Rac1 in basal epidermis was causative in producing skin lesions. Overexpression of Rac1 in human keratinocytes grafted as skin equivalents to SCID mice produced mild epidermal hyperplasia without inflammation, raising the possibility that the immune system could contribute to the phenotype. In summary, these studies suggest that V12Rac1 overexpression in basal epidermis of immune competent mice may be a useful model for studying hyperproliferative skin disorders such as psoriasis.

0717

Global comparison of genes expressed in skin, cultured keratinocytes and artificial skin equivalents, using large DNA microarrays

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Cultured keratinocyte monolayers and skin equivalents are widely used as surrogates for skin in basic and applied research, but a systematic comparison of physiological processes and genes expressed in the three systems has not been done. To identify the similarities and differences between expressed genes in skin, skin equivalents and cultured keratinocytes, we used large oligonucleotide microarrays to derive comprehensive transcriptional profiles. Five independent replicates of each system, 15 microarrays total, yielded extensive, statistically consistent data. We used self organizing maps, correlation clustering algorithms and simple 2-fold difference in expression to identify the differentially expressed genes. The three methods identify largely overlapping lists of differentially expressed genes. As expected, skin and skin equivalents were similar to each other, while keratinocyte cultures differed. Cultured cells do not express epidermal differentiation markers, while expressing a much higher number of cell cycle and DNA replication genes. Skin, in comparison with cultured cells and skin equivalents, expressed a higher number of receptors, secreted proteins and transcription factors, perhaps reflecting the presence of cell types absent from monocultures. Unexpectedly, mitochondrial proteins were severely suppressed in skin. Interestingly, Bcl2 interacting apoptotic proteins are suppressed in keratinocyte cultures, leading to the hypothesis that these proteins effect the biochemical processes that epidermal differentiation and apoptosis have in common.

0718

Estrogens modulate epidermal keratinocyte cell function

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Decreased serum estrogen levels in postmenopausal women is associated with dermal thinning and slower wound healing, however, the exact role of estrogens in the function of keratinocytes remains unclear. The objective of this study is to investigate the mechanism by which estrogens modulate keratinocyte cellular function. Normal human keratinocytes (Kc) express the ERα and ERB and their levels are modulated by physiologic concentrations of estradiol (E_2) (10^{-8}-10^{-11}M). Studies on ^{3}H-thymidine incorporation showed that that E2 (10^{-10}M) stimulates Kc proliferation. This estrogen-mediated effect was abrogated by treatment with pure anti-estrogen (ICI 182780, 10^{-7}M). To investigate estrogen modulation of skin keratinocytes, cells were maintained in growth factor free medium and stimulated with E_2 (10^{-10}M). Cells were harvested and expression of *c-fos* and *c-jun* mRNA were determined by northern blotting. *c-fos* and *c-jun* mRNAs were maximally induced above diluent control 250% and 200% respectively, within 1 hour. Western blotting displayed maximal 150% induction of cyclin D1 protein within 4 hours. Further, within 30 min of E_2 administration tyrosine phosphorylation of the cytoplasmic extracellular signal-regulated kinases 1 and 2 (ERK) were observed with maximal phosphorylation 1 hr after stimulation. Epidermal growth factor receptor mRNA was also induced the by 160% and 300%, respectively after addition of E_2 (10^{-10}, 10^{-11}M). This induction, however, was observed only after 48 hrs, consistent with an indirect effect of estrogen. Our results suggest that in Kc, E_2/ER complex transactivates genes via the classic pathway and stimulates cytoplasmic signal transduction pathways. Thus, modulating estrogen signaling in skin may improve cutaneous function in post-menopausal women.

0719

Reactive oxygen generation is required for actin rearrangements and EGF receptor signaling in migrating keratinocytes

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Reactive oxygen species (ROS) are now recognized as second messengers participating in normal cellular signaling pathways. We asked whether ROS are important in the signaling pathways that control human epidermal keratinocyte migration. Keratinocytes were plated on a collagen I substrate and incubated with N-acetyl cysteine (NAC) to decrease intracellular ROS levels. Cells were imaged using time-lapse photomicroscopy, and the migratory speeds of the cells analyzed. NAC-incubated keratinocytes showed a 25% reduction in migrational velocity compared to keratinocytes maintained in control medium. Effects of NAC treatment were also examined in a wounded monolayer assay, wherein a scratch is made in a confluent monolayer of keratinocytes, and migration of cells from the wound edge to "heal" the wound is monitored by time-lapse photomicroscopy. NAC-treated wounded keratinocyte monolayers showed over a 3.6-fold reduction in the rate of healing 17 h post-wounding compared to a control wounded monolayer. NAC-induced impairment of healing was reversible, and NAC-treated cultures healed within 60 h of NAC removal. Cytoskeletal morphology was also affected by the NAC treatment. NAC-treated cells demonstrated an absence of lamellipodia, as well as increased cortical actin, stress fibers, and large, vinculin-positive focal adhesions. Immunoblot analysis revealed that NAC treatment of keratinocyte monolayers decreased EGF receptor (EGFr) tyrosine phosphorylation 4-fold. Since phosphorylation of the extracellular signal-regulated kinase (ERK) 1/2 occurs as a downstream event in the EGFr pro-migratory signaling cascade, we evaluated ERK1/2 phosphorylation in NAC-treated wounded cultures. NAC treatment of keratinocyte monolayers reduced the activation of ERK1/2 by 2- to 3-fold at 5 and 10 min post-wounding. We conclude that ROS are important second messengers in the EGFr signaling pathway leading to keratinocyte migration. Depletion of ROS results in a blunted EGFr signaling response, leading to altered cytoskeletal morphology and reduced migrational speed.

0720

Novel regulatory mechanism of keratinocyte migration: PP2A activation by β2-adrenergic receptor agonists

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Activation of the β2-adrenergic receptor (β2AR) in keratinocytes inhibits their cell migration by decreasing the phosphorylation of the pro-migratory signaling component, the extracellular-signal-related kinase ERK. Here we demonstrate that these effects are mediated by theβ2AR-induced activation of the serine/threonine phosphatase PP2A. Pre-treating cultured human neonatal keratinocytes with the PP2A inhibitor, okadaic acid (OA, 10nM), completely reverses β2AR agonist-induced retardation of keratinocyte migratory speed as well as β2AR agonist-induced inhibition of wound healing of a confluent sheet of keratinocytes. OA pre-treatment also prevents the β2AR-induced reduction in ERK phosphorylation, implicating PP2A in these processes. Since signaling components are often assembled into intracellular complexes, we explored whetherβ2AR activation could induce the intracellular association of β2AR with other signaling components. Stimulation of keratinocytes with the β2AR agonist isoproterenol results in a rapid association of β2AR with PP2A, as well as a 43% increase in association of PP2A with ERK. This suggests that macromolecular complex formation induced by β2AR activation facilitates PP2A activation and regulates downstream ERK activity. Finally, we find that β2AR activation results in a rapid (30 second) and transient (returning to baseline by 10 min) 2-fold increase in PP2A activity in keratinocytes. Since PP2A is inactivated by phosphorylation of its catalytic subunit, we reasoned that β2AR activation may modulate the phosphorylation state of PP2A. Indeed, we found that β2AR activation reduces by 93% the phosphorylation of the pool of PP2A that is specifically associated with, and co-precipitates with ERK. Thus, this study provides the first evidence that β2AR activation in keratinocytes modulates migration via a novel pathway utilizing PP2A to mediate ERK phosphorylation and alter the pro-migratory signaling pathway. Exploiting this signaling pathway may result in novel therapeutic approaches for control of cell migration.

0721

Activation of phospholipase C-γ1 by c-Src-phosphorylated phosphatidylinositol 3-kinase is required for calcium-induced keratinocyte differentiation

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In normal human keratinocytes, calcium induces the protein synthesis of phospholipase C-γ1 (PLC-γ1) which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to the second messengers inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). These messengers then promote the release of calcium from intracellular stores and the activation of protein kinase C, respectively, which trigger keratinocyte differentiation. To determine whether the activation of PLC-γ1 plays a role in mediating keratinocyte differentiation, we first examined the effect of Src family kinase inhibitor PP2 on these events. We found that the treatment of cells with PP2 prevented calcium-stimulated PLC-γ1 activity and induction of the keratinocyte differentiation markers involucrin and transglutaminase. The increase in PLC-γ1 activity was seen as early as 5 minutes after calcium administration. The increase in PLC-γ1 activity was accompanied by an increase in the protein level and the enzyme activity of c-Src with minimal changes in Fyn. However, unlike the activation of PLC-γ1 by EGF, calcium activation of PLC-γ1 was not a result of tyrosine phosphorylation, suggesting that PLC-γ1 is activated by c-Src indirectly and through a non-phosphorylation mechanism such as phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 is generated by phosphatidylinositol 3-kinase (PI3K). To investigate this mechanism, we examined the effect of PI3K inhibitors on PLC-γ1 activity and keratinocyte differentiation. Our results showed that the PI3K inhibitors LY294002 and wortmannin blocked the induction of keratinocyte differentiation markers as well as PLC-γ1 activity by calcium. Calcium induced the phosphorylation of p85α subunit of PI3K and the activities of p110 subunits of PI3K, mainly γ and δ subtypes. These data provide support that the activation of PLC-γ1 by PI3K, which is phosphorylated by c-Src, is required for calcium-induced keratinocyte differentiation presumably by increasing the level of PIP_3.

0722

EGFR-integrin crosstalk is regulated by endogenous changes in ganglioside expression

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Endogenous modulation of ganglioside GM3 expression affects EGFR signaling through a direct interaction of GM3 with the EGFR; however, GM3 is not able to interact with and regulate α5β1 signaling directly. We hypothesized that ganglioside modulates ligand-independent inhibition of the EGFR signaling by suppressing the complexing and resultant crosstalk between integrin α5β1 and the EGFR. Endogenous levels of ganglioside GM3 were modulated in keratinocyte-derived SCC12 cells by antisense treatment (increased GM3) or by treatment with PPPP or transfection with ganglioside-specific sialidase cDNA (ganglioside depletion). Cells were then starved of EGF and serum, and plated on fibronectin (FN) or plastic. Endogenous accumulation of GM3 inhibited proliferation, whereas ganglioside-depletion stimulated cell proliferation, regardless of growth in the presence of EGF ligand or starved of EGF but in the presence of FN. Similarly, GM3 overexpression inhibited integrin-dependent EGFR activation, while ganglioside depletion enhanced integrin-dependent EGFR activation in the presence of FN. Inhibition of EGFR or MAPK activation and treatment with FAK antisense oligonucleotides further suppressed the phosphorylation of EGFR, FAK, and MAPK. Incubation with cytochalasin D also further suppressed the phosphorylation of FAK and MAPK. These studies of signaling pathway inhibition provide evidence that the effect of ganglioside occurs upstream, probably at the membrane level. EGFR and α5β1 are both found in membrane immunoprecipitated with either anti-EGFR or anti-α5β1 antibody; endogenous overexpression of GM3 eliminated the co-immunoprecipitation, while depletion of ganglioside augmented it, suggesting that the mechanism of the ganglioside-induced inhibition of crosstalk involves blockade of complexing of the EGFR with integrin. These studies show that endogenous modulation of ganglioside expression affects EGFR-integrin crosstalk, at least in part by preventing association of EGFR and integrin α5β1, and may be a powerful tool for regulating epithelial cell proliferation and cell motility.

0723

9-O-acetylation of ganglioside GD3 may promote the hyperproliferative phenotype of keratinocytes in psoriasis and cutaneous carcinoma

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Ganglioside 9-O-acetylGD3, an acetylated derivative of GD3, is significantly expressed in psoriatic epidermis and in cutaneous carcinomas, but is undetectable in normal epidermis. The role of 9-O-acetylGD3 expression in these skin disorders is unknown, although a T-cell costimulatory role has been suggested. We hypothesized that 9-O-acetylation of GD3 promotes epithelial cell hyperproliferation and cell survival, and investigated the mechanisms of these putative effects. We stably transfected a ganglioside-specific 9-O-acetyltransferase into the keratinocyte-derived SCC12 cells, and have evaluated resultant transfectants both in culture and in a mouse tumor model. 9-O-acetyltransferase transfection led to a marked reduction of GD3 expression and increased 9-O-acetylGD3 without significantly depleting GM3. Overexpression of 9-O-acetylGD3 led to significant increases in proliferation of transfected cells in culture, regardless of incubation with serum or in serum-free medium on a fibronectin or vitronectin matrix. Consistently, subcutaneous inoculation of 9-O-acetyltransferase-transfected cells resulted in tumors that were 8-fold larger than control SCC12 cell tumors after 40 days. This increase in proliferation correlated with increased EGFR and MAPK phosphorylation in 9-O-acetyl transferase overexpressors. 9-O-acetylGD3 expression also led to increased cell survival, consistent with the previous demonstration that GD3 triggers apoptosis, regardless of matrix. 9-O-acetyltransferase overexpression activated PKCα without affecting its expression, whereas functional blockade of 9-O-acetylGD3 prevented PKCα activation. These data suggest that shifts in ganglioside expression contribute to the phenotype of hyperproliferation and increased cell survival of psoriatic keratinocytes and cutaneous carcinoma cells.

0724

Induction of apoptosis by p33ING1 via Bcl-2 and caspase-3

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The biological functions of the tumor suppressor ING1 have been studied extensively in the last five years. Four alternatively spliced forms of ING1, p47ING1, p33ING1, p27ING1, and p24ING1 have recently been identified. ING1 has been shown to mediate growth arrest, senescence, apoptosis, anchorage-dependent growth, chemosensitivity, and DNA repair. In addition, it has been shown that ING1 requires the presence of p53 to exert its biological functions. Induction of apoptosis has been demonstrated by adenovirus-mediated transfer of both p33ING1 and p53 in glioma and esophageal carcinoma cells. Apoptosis can also be facilitated by UV irradiation in human fibroblast and melanoma cells expressing p33ING1. To further investigate the molecular pathway by which p33ING1 induces apoptosis, we overexpressed p33ING1 by adenoviral infection in HEK293 cells, and subsequently assayed for the frequency of cell death. Our results, supported by two independent assays, indicated that overexpression of p33ING1 enhanced apoptosis by as much as 70% in HEK293 cells compared to vector controls. We also showed that the expression of p53 decreases with increasing levels of p33ING1. The biological significance of this relationship between p53 and p33ING1 is yet to be determined. Furthermore, other proteins that were reduced in expression were Bcl-2 and procaspase-3, suggesting that the apoptotic pathway regulated by p33ING1 depends on the inhibition of the anti-apoptotic protein Bcl-2 and the activation of caspase-3. Taken together, these results strongly indicate that p33ING1 participates in apoptosis via the Bcl-2/caspase-3 pathway.

0725

Calcium sensing receptor forms a signaling complex with IP3R, PLCγ1 and golgi Ca-ATPase in keratinocytes

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Calcium is critical for regulating keratinocyte differentiation. Raising extracellular calcium concentration above 0.07 mM induces a rapid rise in intracellular free calcium and enhances expression of differentiation marker genes in keratinocytes. We previously reported that the calcium sensing receptor (CaR) is required for mediating these calcium-induced cellular responses. CaR knockdown results in a reduction of calcium release from internal stores. This result implies an interaction between CaR and other calcium regulating molecules, through which CaR regulates internal calcium stores. Double fluorescence immunostaining examined by confocal microscopy showed that CaR colocalizes with IP3R and PLCγ1 in intracellular compartments. To further investigate the possibility of complex formation between CaR and other calcium signaling molecules we performed subcellular fractionation and determined whether these proteins were found in the same fractions. Keratinocyte membranes were subjected to a 15-50% discontinuous sucrose gradient. After high-speed centrifugation, each fraction was collected and analyzed by immunoblotting. Whereas the non-glycosylated CaR was found in the ER/cis-golgi fractions, the mature glycosylated forms of the CaR were found in the trans-golgi fractions. Whereas the ER calcium pump, SERCA2, was found in the ER fraction, ATP2C1, the human homologue of a yeast golgi Ca-ATPase PMR1, as well as other calcium regulating proteins including IP3R and PLCγ1 were found in the trans-golgi fractions. Co-immunoprecipitation studies using antibodies against CaR, IP3R, PLCγ1 and ATP2C1 demonstrated that these antibodies not only precipitated their target proteins but also reciprocally brought down the other three molecules from the trans-golgi fractions. These results indicate that in keratinocytes CaR, IP3R, PLCγ1 and ATP2C1 form an intracellular complex in trans-golgi. Our data suggest that the interactions among these molecules regulate calcium release from intracellular stores.

0726

Visualisation in real time of NFAT2 nuclear translocation induced by differentiation signals in human keratinocytes

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The transcription factor nuclear factor of activated T cells (NFAT) has recently been implicated in both mice and humans and we have previously reported expression of calcineurin and NFAT1 in human keratinocytes. In this study we investigated the subcellular localisation and translocation of NFAT2 using a fusion protein comprising full-length human NFAT2 and green fluorescent protein (GFP) and real time confocal imaging. Reverse transcription/PCR and western analysis demonstrated the presence of NFAT2 in cultured normal human keratinocytes. Following transfection with GFP-NFAT2, keratinocytes were treated with agents that induced a rise in intracellular calcium and visualised by confocal microscopy. The proportion of GFP-NFAT2 within the nucleus was quantified as a function of time. GFP-NFAT2 appeared predominantly cytoplasmic in un-treated keratinocytes although cells with clear nuclear GFP-NFAT2 localisation were also observed. Ionomycin (1 μM) induced nuclear translocation by 3-6 mins that was maximal at 10-20 mins (n=3). TPA (50 nM) and increased extracellular calcium (1.5 mM $[Ca^{2+}]_o$) induced nuclear translocation of GFP-NFAT2 with slower kinetics compared to ionomycin. Pre-treatment of keratinocytes with the phosphatase calcineurin inhibitor cyclosporin A (1 μM) inhibited nuclear translocation of GFP-NFAT2. Interestingly, GFP-NFAT2 was observed to translocate to discrete regions within the keratinocyte nuclei. The nature of these intranuclear bodies remains to be determined but it is intriguing that the size of these bodies increased over time as GFP-NFAT2 accumulated. In summary, these data indicate that NFAT2 subcellular localisation is tightly regulated by calcium and differentiation signals through calcineurin activation and suggests that NFAT2 may play a role in regulating keratinocyte differentiation.

0727

Blockade of the epidermal growth factor receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation

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In chronic inflammatory skin disorders such as psoriasis, atopic dermatitis and allergic contact dermatitis, keratinocytes produce inflammatory mediators and undergo increased proliferation leading to epidermal hyperplasia. Leukocyte-derived TNF-α and IFN-γ are mostly responsible for induction of inflammatory mediators and increased expression of growth factors. Here we investigated the impact of the EGF receptor (EGFR) and the EGF family growth factor TGF-α on TNF-α- and IFN-γ-induced chemokine expression by keratinocytes. Extended epidermal EGFR and TGF-α expression was detected not only in psoriasis but also in chronic atopic dermatitis and chronic allergic contact dermatitis. In cultured human keratinocytes, metalloproteinase-dependent shedding of the mature form of TGF-α was rapidly induced by both TNF-α and IFN-γ, and directly contributed to early EGFR transactivation. When added exogenously, TGF-α dose-dependently down-regulated TNF-α- and IFN-γ-induced expression of CCL2, CCL5 and CXCL10. In contrast, TGF-α synergized with TNF-alphaα for CXCL8 induction. Conversely, impairment of autocrine EGFR-ligand system by the selective EGFR tyrosine kinase blocker PD168393 prior to stimulation with cytokines led to increased CCL2, CCL5 and CXCL10, but reduced CXCL8. In mouse contact hypersensitivity, impairment of EGFR tyrosine kinase activity by a single topical administration of PD168393 prior to hapten challenge induced an exacerbated inflammatory response, which was associated to increased epidermal levels of CCL2, CCL5 and CXCL10, and higher numbers of CD4+ and CD11b+ leukocytes infiltrating the skin. Similar perturbations were obtained with local painting of the MEK1/2 inhibitor PD98059. In conclusion, EGFR signaling may serve as a mechanism to dampen skin inflammation and protect epithelial cells from injury. On the other hand, the blockade of EGFR signaling on epithelial cells may be exploited to enhance inflammatory responses.

0728

Nerve growth factor affects anagen-catagen transformation of cultured human anagen hair follicles

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Our understanding of neurotrophin signaling in many non-neuronal tissues is increasing daily since the discovery of the prototypic nerve growth factor (NGF). Recently, we could show, that brain derived nerve growth factor (BDNF) is playing a crucial role in murine and human hair follicle regression (catagen). We here investigate, presence and function of NGF and its high affinity receptor tyrosinekinase A (TrkA) in human anagen hair follicles by rtPCR, in situ, immunofluorescense and organ culture. NGF and TrkA are expressed in the anagen hair follicle with distinct distribution patterns: NGF is dominant in more differentiated keratinocytes such as the inner root sheath. It is also expressed by the hair matrix, dermal papilla cells, the arrector pili muscle and the sebaceous glands. Outside the follicular compartment NGF can be detected in Langerhans cells, mast cells and nerve fiber bundles. Complementary, TrkA expression is dominant in the more undifferentiated keratinocytes of the outer root sheath with little expression outside the follicular compartment. These observations suggest NGF/TrkA signaling in epithelial growth control in the anagen hair follicle. In fact, NGF did decrease hair shaft elongation in high concentrations. Also, hair follicles cultured with 50ng/ml NGF displayed a progressed catagen-like hair cycle stage. Interestingly, apoptosis of hair follicle keratinocytes did not increase (TUNEL-assay) under NGF treatment, while the number of proliferating (Ki-67-immunoreactivity) keratinocytes did, indicating a higher turn-over rate in the regressing hair bulb. NGF-neutralizing antibodies had no significant effect in this culture model. This suggests, that NGF is playing part in epithelial tissue remodeling during anagen-catagen transformation in the human hair follicle.

0729

Regulation of parathyroid hormone-related protein gene expression by EGF family ligands in primary human keratinocytes

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Cultured primary human keratinocytes were the first non-cancer derived cell type reported to produce the humoral hypercalcemia factor, PTHrP. Normal keratinocytes produce more PTHrP than some squamous cell carcinoma cell lines. Emerging evidence suggests that only a subset of keratinocytes produce high levels of PTHrP in vivo. It is likely that conditions associated with growth in culture activate PTHrP gene expression in primary keratinocytes. In culture, keratinocytes produce a number of EGF-like ligands, as well as express the receptors for these ligands, the erbB family receptors. To investigate the impact of erbB receptor signaling on PTHrP gene expression, primary human foreskin keratinocytes were treated with inhibitors of erbB1 (PD 153035) or erbB2 (AG 825) and subsequently stimulated with EGF. PTHrP gene expression was then assessed by quantitative RT-PCR, RNase protection, and reporter gene assays. Stimulation of erbB1 by exogenous EGF or endogenously produced EGF-like ligands accounts for more than 90% of PTHrP mRNA produced by rapidly growing primary keratinocytes. In differentiated keratinocytes, both erbB1 and erbB2 signaling stimulates PTHrP gene expression. ErbB-family dependant activation of PTHrP expression was due to increased transcription of the PTHrP gene. This induced transcriptional activity was due to the activation of the PTHrP P3 promoter and required the Ets transcription factor-binding site. Future experiments will identify the specific ErbB1 ligand, second messenger pathway and Ets factor responsible for stimulation of PTHrP gene expression in human keratinocytes. These findings suggest EGF-like ligand expression, overexpression of erbB receptors and the acquisition of specific Ets factors may stimulate PTHrP production in hyperproliferative epidermal conditions as well as epidermal tumors.

0730

Interleukin-1 augments UV-induced apoptosis by different mechanisms

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Treatment of KB cells with the death ligands FasL or TRAIL and irradiation with UVB, respectively, induces apoptotic cell death. Prestimulation of KB cells with interleukin-1 (IL-1) which activates the transcription factor nuclear factor kB (NFkB) was shown to protect cells from FasL- and TRAIL-induced apoptosis via upregulation of the inhibitor of apoptosis proteins (IAP) c-IAP and x-IAP. In contrast, UVB- induced apoptosis was significantly enhanced upon prestimulation with IL-1 and coincided with a strong NFkB-mediated release of the proapoptotic cytokine tumor necrosis factor a (TNFa) in the range of 100 pg/ml. No TNFa release occurred upon treatment of KB cells with IL-1 plus either one of the death ligands. Surprisingly, costimulation of cells with 100 pg/ml TNFa and UV resulted only in a rather weak enhancement of UV-induced apoptosis, indicating that other mechanisms besides TNFa release have to be involved in the apoptosis enhancing effect of IL-1. Semiquantitative PCR analysis revealed that NFkB-dependent transcription of c-IAP and x-IAP as well as of FLIP (FLICE inhibitory protein), another anti-apoptotic protein, was significantly downregulated by UV. This downregulation seemed to be critically dependent on UV-induced DNA-damage, since it could be reversed by addition of liposomes containing the DNA repair enzyme photolyase. Addition of IL-1 to UV-exposed cells caused a further downregulation of the antiapoptotic proteins, thus explaining the enhanced death rate of UV-exposed cells in the presence of IL-1. Together, these data demonstrate that IL-1 enhances UV-induced apoptosis in several ways, first by inducing the secretion of TNFa and second by supporting the downregulation of antiapoptotic proteins. This further indicates that the combination of IL-1 and UV can cause transcriptional up- (TNFa) and downregulation (antiapoptotic proteins) of different genes in parallel and thereby contribute to the same biological effect, i.e. enhancement of apoptosis.

0731

IL-6-type cytokines up-regulate expression of multi-drug resistance associated proteins in NHEKs and dermal fibroblasts

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Normal human epidermal keratinocytes (NHEK) and dermal fibroblasts have been shown to express a cell-type specific pattern of influx and efflux transport proteins as well as extrahepatic cytochrome P450 enzymes showing that these cells metabolize and excrete a variety of xenobiotics like drugs. In this project we analysed the influence of IL-6-type cytokines on the expression of multidrug resistance associated proteins (MRP1-5). Using RT-PCR, real-time PCR, cDNA microarrays and calcein acetoxymethyl ester transportassays we were able to show that stimulation of NHEKs, HaCat keratinocytes or human dermal fibroblasts with IL-6 in combination with its soluble IL-6 receptor (sIL-6R) or Oncostatin M for 48 hours resulted in an upregulation of MRP-expression and activity. Incubation of NHEKs with 20ng/ml IL-6 and 1 µg/ml sIL-6R for 48h revealed the upregulation of MRP1, MRP3, MRP4 and MRP5 expression on the mRNA-level. Efflux activity of these cells was increased by 70%. Expression of these genes was stronger upregulated in HaCat keratinocytes. Incubation of dermal fibroblasts with IL-6/sIL-6R resulted in a 40% upregulation of MRP3 expression, efflux activity was upregulated by 60%. Incubation with Oncostatin M even resulted in a nearly 100% upregulation of MRP3 expression in these cells. It is well known that several inflammatory skin diseases like psoriasis show an enhanced expression of IL-6-type cytokines. Therefore the upregulation of efflux drug transporters by these cytokines may have an important influence on pathogenesis and treatment of these diseases.

0732

The p75 neurotrophin receptor modulates apoptosis and differentiation in a subpopulation of human keratinocytes

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The p75 neurotrophin receptor (p75NTR) belongs to the TNF-receptor superfamily and signals apoptosis in many cell settings. We have shown previously that human keratinocytes express p75NTR, although its role has never been investigated. The aim of the present study was to analyze the pattern of expression and the function of p75NTR in human keratinocytes. First, p75NTR is localized in a minor subpopulation of basal keratinocytes as well as in suprabasal cells, with an irregular and patchy pattern. Second, p75NTR mRNA is more expressed in keratinocytes slowly adhering (transit amplifying cells, TA) than in keratinocytes rapidly adhering (stem cells, KSC) to type IV collagen. % p75NTR positive cells was higher in TA than in KSC, as evaluated by immunohistochemistry in situ. Moreover, p75 protein was more expressed in confluent than in subconfluent keratinocytes, as shown by western blot. BDNF, which signals through p75NTR in keratinocytes, induces cell cycle arrest in G2M. Moreover, BDNF (50-75-100 ng/ml) reduces keratinocyte growth in a dose- and time-dependent (24-48-72 hrs) manner, as shown by the MTT assay (50 ng/ml, $p < 0.05$). BDNF also induces keratinocyte apoptosis in a dose-dependent fashion (100 ng/ml, $p<0.05$ at 48 hrs), as shown by TUNEL. Anti-p75 neutralizing antibody partially inhibits the BDNF-induced keratinocyte cell death. Finally, p75NTR expression completely disappears in sections from psoriatic lesions. We speculate that p75NTR modulates apoptosis and possibly differentiation in a subpopulation of keratinocytes

0733

Inhibition of the IFNγ-mediated Jak-STAT signaling pathway by iron chelators occurs via down-regulation of the IFNγ receptor-1

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Interferon-gamma (IFNγ), a cytokine important in inflammation and cell cycling, mediates its effects by binding to IFNγ receptor complex and activation of the Jak-STAT signaling pathway. Activation of this cascade results in nuclear translocation of phosphorylated STAT1 homodimers that bind to GAS motifs in the promoters of target genes, such as interferon regulatory factor 1 (IRF-1). Prior studies by our laboratory have identified that IFNγ dependent transcription of IRF-1 in dermal endothelial cells (HDMEC) requires iron. In this study, we investigated whether activation of the Jak-STAT signaling pathway, an essential step in IFNγ mediated IRF-1 gene transcription, requires iron. IFNγ treatment of HDMEC resulted in STAT1 activation and nuclear translocation as assessed by electrophoretic mobility shift assay and supershift studies using IRF-1 promoter based oligonucleotides. Pretreatment of HDMEC with the iron chelator dipyridyl (DP) inhibited IFNγ mediated complex formation in a concentration- and time-dependent fashion. In contrast, DP pretreatment had no effect on TNFα mediated activation and translocation of NF-kB. To assess if DP-mediated inhibition of STAT1 DNA binding was due to decreased STAT1 activation, we measured levels of total and phosphorylated STAT1 protein by western analysis. IFNγ induced STAT1 phosphorylation was almost completely inhibited by DP pretreatment, while not affecting total cellular STAT1 protein levels. Removal of iron did not affect the expression of Jak1, Jak2, or IFNγ receptor 2, which are all necessary for IFNγ mediated STAT1 activation. However, DP pretreatment downregulated the expression of IFNγ receptor-1 (IFNGR1) protein, the critical receptor chain mediating IFNγ ligand binding and Jak activation. These data provide evidence that iron is essential for IFNγ mediated cell signaling via iron dependent expression of IFNGR1 and define a novel link between iron and immune function.

0734

Global transcriptional responses of human keratinocytes to heat shock

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While most cell types react in vitro to heat stress at the molecular level by activating a "heat-shock response", only the epidermal keratinocytes are the commonly a target of burns in humans in vivo. To define the responses of human keratinocytes to heat-shock, we used large oligonucleotide microarrays that can measure transcription of 12,000 genes at once, and probed them with mRNAs from heat-treated and control keratinocytes. We tested four time points, 1, 4, 24 and 48 hrs post-treatment; each with a corresponding, untreated control. As expected, the heat-shock proteins are induced, but we also observed in human keratinocytes a significant induction of genes involved in DNA repair, cell cycle, the inositol phosphate pathway and coding for cytochromes. In contrast, the cornified envelope components, translation factors and many cytoskeletal and nucleoskeletal proteins were suppressed, as were the Ras and Rho pathway components. Interestingly, a large number of cell surface receptors, as well as secreted protein factors are regulated by heat-shock, suggesting that keratinocytes alert the surrounding tissue to the occurrence of heat damage. From these results, we conclude that keratinocytes, when damaged by heat, elaborate an extensive program of self-protection and repair, but also alert the underlying tissue that a burn has occurred.

0735

Overexpression of wildtype transforming growth factor β1 (TGFβ1) in the epidermis of transgenic mice results in phenotypes that mimic psoriasis

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Psoriasis is an inflammatory skin disorder. Overexpression of TGFβ1, a growth inhibitor for keratinocytes, has been found in psoriatic epidermis. This has been assumed to be a negative feedback that counteracts with increased growth factors in psoriasis. We have developed transgenic mice overexpressing wildtype TGFβ1 driven by a keratin 5 promoter (K5.TGFβ1^{wt}). Surprisingly, these mice displayed phenotypes that resembled psoriasis at macroscopic and pathological levels. Besides exhibiting major characteristics of psoriasis, i.e., epidermal hyperplasia, inflammation and angiogenesis, K5.TGFβ1^{wt} skin showed additional psoriatic phenotypes that have not been found in other mouse models for psoriasis. These include Koebner's sign, basement membrane degradation and microabcesses. Among these phenotypes, matrix metalloproteinases-mediated basement degradation appeared to be a key step towards a psoriasis-like inflammation in transgenic skin. Furthermore, K5.TGFβ1^{wt} skin exhibited molecular changes that usually occur in psoriasis. IL-1, the primary pro-inflammatory cytokine, IL-8 (MIP-2 in murine), a hallmark chemokine in psoriasis, and several other chemokines were upregulated in K5.TGFβ1^{wt} skin. Increased angiogenesis accompanied by the elevated expression of vascular endothelial growth factor and its receptor were found in transgenic skin. Moreover, multiple kerationcyte growth factors were elevated in the dermis of transgenic skin. These include keratinocyte growth factor and insulin-like growth factor-1. Elevation of these growth factors by the paracrine effect of TGFβ1^{wt} apparently overcame the growth inhibitory effect of the TGFβ1 transgene on keratinocytes and led to epidermal hyperplasia and hyperproliferation in K5.TGFβ1^{wt} skin. Taken together, we provide evidence that TGFβ1 overexpression functions as an initiator and coordinator in the development of a psoriasis-like skin disorder.

0736

Significance of immunohistochemical determination of HER-2/neu, CD117 (c-Kit) and vascular endothelial growth factor expression in malignant melanoma

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HER-2/neu, a 185kd oncoprotein, is a recently identified therapeutic target in several solid tumors. Similarly, currently, there exist target-directed therapies against CD117 (c-Kit) and vascular endothelial growth factor (VEGF), both of which likely play a role in melanogenesis. To evaluate the role of HER-2/neu, c-Kit and VEGF in malignant melanoma, we studied individual oncoprotein overexpression in 202 subjects. An extensive chart review was performed to obtain demographic and clinical data. In addition, overexpression of HER-2/neu (using the DAKO Hercep test), c-Kit (using the rabbit polyclonal antibody A4502) and VEGF (utilizing the avidin-biotin-complex method) was evaluated on paraffin-embedded tissue samples. 109 males (54%) and 93 females (46.0%) with a mean age of 57 years (range: 15-101 years) were included in the study. Of the 202 specimens, only 2 (0.9%) revealed HER-2/neu overexpression, whereas 46 (22.8%) overexpressed c-Kit, and 42 (20.8%) specimens showed VEGF overexpression. Multivariate analysis (after adjusting for age, sex, EC0G score, Breslow thickness, stage and therapy) did not show a survival difference between the c-Kit positive and negative groups (p=0.36), and likewise between the VEGF positive and negative patients (p=0.25). Interestingly however, c-Kit was more likely to be overexpressed in the superficial spreading type (63.0% of c-Kit positive samples). In contrast, VEGF was overexpressed preferentially in amelanotic melanoma (33.3% of VEGF positive specimens). From our large study, it is evident that HER-2/neu has no role in melanogenesis or melanoma therapy, and, despite the lack of prognostic value, since 43.6% of melanoma specimens revealed either c-Kit or VEGF expression, the therapeutic role of anti-VEGF monoclonal antibody therapy and/or signal transduction inhibition using tyrosine kinase inhibitors warrants further evaluation.

0737

Effects of spingosine-1-phosphate on apoptosis of B16 melanoma cells

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The bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) is the ligand for a family of five G protein-coupled receptors, the Edg (endothelial differentiation gene) receptors, which includes Edg -1, 3, 5, 6, and 8. Activation of these receptors by S1P regulates diverse processes, including cell migration, angiogenesis, vascular maturation, heart development, and neurite retraction. A few studies of the relationship between S1P and melanoma cells have been reported. They reported that S1P inhibited motility of cultured melanoma cells. Melanocytes and melanoma cells are known to be of neural origins. The effects of S1P on apoptosis of neural cells are diverse depending on the cell line. There has been no report on the effect of S1P on melanoma cell growth. In present study, we investigated in vitro the effects of S1P on B16 melanoma cell proliferation. For the first time, we confirmed the expression of Edg-1, 3, and 5 on cytoplasm of B16 melanoma cells by Western blot and immunocytochemistry methods. We observed the inhibitory effect of S1P on proliferation of B16 melanoma cells by in vitro culture in a dose-dependent pattern. We also observed that S1P at a dose of 10 μmol induced apoptosis of B16 melanoma cells by TUNEL assay. Currently we are researching whether S1P induces the activation of caspases and JNK in order to confirm the signaling pathway of apoptosis induced by S1P in B16 melanoma cell line.

0738

Involvement of extracellular regulated protein kinase in fibroblast by interleukin 6

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Keloids are characterized by the proliferation of fibroblasts and an increased accumulation of extracecellular matrix, but no specific etiology has been identified for the pathogenesis of this disorder. Interleukin-6 (IL-6) is an important immunoregulatory cytokine that has been implicated in a number of fibrotic autoimmune diseases such as scleroderma, interstitial nephritis, and pulmonary interstitial fibrosis. However, the role of IL-6 in the development of keloids is still unclear. In this study, keloid fibroblasts and NIH3T3 fibroblasts were stimulated by IL-6 in the presence or absence of PD98059, a specific MAPK/ERK kinase-1 inhibitor, and proliferation rate was examined. The results showed that IL-6, at dose of 60 ng/mL and 10 ng/mL, stimulated proliferation in keloid and NIH3T3 fibroblasts, associated with induction of ERK1/2 phosphorylation. Pretreatment of fibroblasts with PD98059 showed dose-dependent inhibition on IL-6-induced proliferation and MAPK phosphorylation. However, p38 and JNK proteins were remained unchanged in IL-6 treated cells. These results demonstrated that phosphorylation of ERK proteins were involved in IL-6-induced proliferation, and suppression of MAPK phosphorylation might be beneficial in treatment of keloid.

0739

Protein kinase C alpha associates with phospholipase D1 and enhances both basal phospholipase D activity and invasive potential in a protein phospholylation-independent manner in human melanoma cells

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It is well known that phospholipase D (PLD) plays a crucial role in the signal transduction of many types of cells, and is activated by protein kinase C alfa (PKC alfa) when cells are stimulated. To elucidate the role of PLD in melanoma,the mechanism of regulation of basal PLD1 activity by PKC alfa was examined in a human melanoma cell line HM3KO using an adenovirus-mediated gene transfer technique. Immunoprecipitation study showed that PLD1 and PKC alfa are associated physiologically in resting melanoma cells. Further immunoprecipitation study after adenovirus-mediated simultaneous overexpression of PLD1 and PKC alfa, or PLD1 and the kinase-negative mutant of PKC alfa revealed that both PKC alfa and the kinase-negative mutant of PKC alfa are associated with PLD1 in melanoma cells in the absence of an external signal. Overexpression of PKC alfa or the kinase-negative mutant of PKC alfa in melanoma cells by the adenovirus vectors resulted in the enhancement of basal PLD activity in a viral dose-dependent manner. Furthermore, enhanced basal PLD activity increased the in vitro invasive potential of HM3KO cells. These results suggest that PKC alfa associates with PLD1 and enhances basal PLD activity in a protein phosphorylation-independent manner via direct protein-protein interaction in melanoma cells, which contributes to high invasive potential of cells.

0740

Keratinocyte growth factor receptor is expressed in proliferating HaCaT cells and is down-regulated by dithranol

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Keratinocyte growth factor (KGF) is a paracrine modulator of keratinocyte growth. Both KGF and its receptor, KGFR shows elevated levels of mRNA and protein expression in the hyperproliferative skin disease, psoriasis. We studied the expression of KGFR in relation with keratinocyte proliferation and differentiation using synchronized HaCaT cell model system. The effect of dithranol on KGFR expression was also investigated. HaCaT cells were synchronized by contact inhibition and serum starvation then released from cell quiscence by passage and addition of serum. Using reverse transcription PCR and Western blot techniques KGFR mRNA as well as protein levels were determined in the cultured cells after moved from cell quiscence in the cell cycle and started to differentiate. Reverse transcription PCR reactions showed that KGFR mRNA expression considerably increased in the proliferating cells. At the same time KGFR protein was not detectable in differentiated only in proliferating HaCaT cells. These results suggest that KGFR expression is regulated both at the transcriptional and post-transcriptional levels in HaCaT cells. Treatment with the different concentrations of dithranol resulted in a dose-dependent decrease of KGFR both at the mRNA and protein levels, suggesting that down-regulation of KGFR may be part of the effect of this antipsoriatic agent.

0741

IGF-II-mediated expression of angiogenetic factors VEGF and IL-8, and COX-2 in human keratinocytes

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Cytokines upregulated in psoriasis include IGF-II, IL-6, IL-8, TGF-a and VEGF, which are presumably associated in the pathogenesis of psoriasis. To examine the relationship between the cytokines involved in the angiogenesis, we monitored the expression of VEGF and IL-8 as well as an inflammatory factor COX-2 in the IGF-II treated human keratinocytes and explored the IGF-II signaling pathways with respect to the expression of VEGF, IL-8 and Cox-2. IGF-II induced VEGF and Cox-2 at the levels of mRNA and protein not only in the primary keratinocytes but also in HaCaT cells. The elevated expression of VEGF, however, was abrogated when pretreated with inhibitors of tyrosine kinase, src and ERK, respectively, while inhibitors of PI3 kinase and PKC blocked to a less degree the IGF-II mediated VEGF expression and the p38 inhibitor did not. Up-regulation of COX-2 by IGF-II also disappeared in the cells pretreated with inhibitors of tyrosine kinase, src, PI3 kinase and PKC, respectively. Electrophoretic mobility shift assay demonstrated that the nuclear extracts from the IGF-II treated cells contained enhanced DNA binding activities for the NF-kB and the AP-1 probes. Blocking the DNA binding activities of NF-kB and AP-1 diminished the levels of the IGF-II stimulated induction of VEGF and COX-2. Moreover, the IL-8 induction by IGF-II was abolished by inhibitors of tyrosine kinase, src, ERK and PI-3 kinase, respectively. These results suggest that the induction of VEGF, IL-8 and COX-2 by IGF-II in the human keratinocytes may involve two signaling pathways: the tyrosine kinase-src-ERK-AP-1 pathway and the PI3 kinase pathway.

0742

The ultraviolet-B radiation-induced inhibition of interferon-gamma caused keratinocyte activation is independent of endogenous interleukin-10 and other soluble mediators but associated with enhanced intracellular suppressor of cytokine signaling expression

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Ultraviolet irradiation represents a well established treatment modality for inflammatory skin diseases. The aim of this study was to investigate the mechanisms of UVB radiation (UVBR) induced KC insentitivity towards IFN-g. Flow cytometric analyses indicated that UVBR temporarily inhibits the IFN-g-caused activation of primary keratinocytes and HaCaT cells as measured by reduced ICAM-1 and HLA-DR upregulation. Western blot experiments suggested that this is mediated by the UVBR-induced inhibition of STAT-1 phosphorylation. Neither IL-10 neutralization nor IL-10 addition had any effect on the UVBR-induced inhibition of IFN-g caused ICAM-1 expression on primary keratinocytes or HaCaT cells. Furthermore the supernatant from UVB-irradiated cells failed to inhibit the IFN-g-mediated ICAM-1 and HLA-DR upregulation in non-radiated HaCaT cells. Moreover irradiated cells from whom the supernatant was withdrawn 4 hours after irradiation still showed a diminished IFN-g-induced response after 24 hours. Thus no soluble but intracellular factors might be involved in the UVBR-induced inhibition of IFN-g-caused KC activation. Therefore we analyzed the expression of members of the intracellular protein family of suppressors of cytokine signaling (SOCS) molecules using a real-time PCR approach. We found a strong upregulation of SOCS 1 after UVBR with peak levels at 1 hour. Since SOCS molecules are known inhibitors of STAT phosphorylation that is essential for IFN-g-induced ICAM-1 and HLA-DR upregulation, this may explain the IFN-g unresponsiveness after UVBR.

0743

Evidence that nitric oxide is an upstream mediator of FasL-mediated keratinocyte apoptosis in toxic epidermal necrolysis

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Toxic epidermal necrolysis (TEN or Lyell's syndrome) is a severe adverse drug reaction that results in extensive necrolysis of the skin due to large scale Fas-FasL mediated keratinocyte apoptosis. The signaling pathways leading to the rapid upregulation of keratinocyte FasL expression in TEN are unknown. Here we show that FasL and inducible nitric oxide synthase (iNOS), an enzyme that leads to the production of the proinflammatory and cytotoxic molecule nitric oxide (NO), are coexpressed in increased amounts by keratinocytes in lesional skin samples of TEN. In vitro, within 8 h of exposure of keratinocytes to NO donors (NOC18 or SNP), a significant increase in FasL mRNA levels and cell surface FasL could be detected. Cell surface FasL induced by NO donors was lytically active, as within 8h both NOC 18 and SNP caused a 4-fold increase in keratinocyte apoptosis. The latter was completely abolished by the caspase-8 inhibitor ZVAD, and significantly reduced by Fas:Fc confirming that NO triggers rapid FasL-mediated keratinocyte cell death in vitro. Similarly, in an in vitro model of human skin (EpiDerm TM, MatTek Corp.), NOC18 induced keratinocyte FasL expression, keratinocyte apoptosis and histopathological modifications reminiscent of those observed in early biopsy specimens of TEN. In this model, keratinocyte apoptosis could be virtually completely inhibited by the caspase-8 inhibitor Z-IETD-FMK. Taken together, our data show that iNOS via the action of NO is likely to be an upstream trigger of FasL-mediated keratinocyte apoptosis in TEN.

0744

Calcium (Ca^{++}) signaling through a novel Ca^{++}-binding protein in mouse keratinocyte differentiation

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The Ca++ signaling dependent systems, such as keratinocyte differentiation process, must be responsive to rapid and transient variations in Ca++ concentration. A central role in the transduction of Ca++ signals is played by members of the Ca++-binding proteins. Calmodulin (CaM) is the best studied of these signaling proteins that function as calcium sensors. Members of this subfamily are small, acidic (PI 4-5) proteins that contain EF-hand motifs that bind Ca++. It is through the binding of Ca++ by the EF-motifs that these sensor proteins are able to bind or liberate their target interacting proteins and in this way modulate their function. Performing a Suppressive Subtractive Hybridization (SSH) screen we obtained a partial cDNA clone (called B12) that has homology to CaM-like proteins. Using a RACE protocol and a gene-specific oligonucleotide, we obtained the full-length B12 cDNA. The ORF encodes a protein of 148 amino acid residues with a PI of 4.59, predicted MW of 16.8 kDa and contains four conserved EF-hand motifs, predicted to be Ca++-binding domains. The deduced amino acid sequence had 56% homology with mouse CaM. Northern blot and in situ hybridization analysis showed restricted differential expression of the B12 gene in the suprabasal layers of the epidermis. Furthermore, induction of B12 expression was observed upon in vitro differentiation by Ca++ of primary cultured keratinocytes. The functionality of B12 EF-hand domains was assayed with a radioactive Ca++-binding method, and the quantitated data shows that B12 binds Ca++ ions 6.2 times more the CaM positive control used in the experiments. Therefore, our preliminary data indicates that the B12 EF-hand motifs are functional Ca++-binding domains. Ca++-binding proteins and CaM exert their role through specific interaction with their target proteins. To identify B12 target protein we are performing a yeast II hybrid screen. Identification of target proteins will help us understand the specific role of B12 during keratinocyte differentiation.

0745

Centaurin β ADP-Ribosylation Factor GTPase Activating Proteins identify novel cell membrane domains in primary cultured keratinocytes

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The ADP-Ribosylation Factor family of small guanine nucleotide binding proteins control membrane trafficking events and regulate actin cytoskeleton mediated changes in cell morphology. β-centaurins are multidomain proteins containing ARF-GTPase Activating Protein and phosphoinositide binding Pleckstrin Homology (PH) domains. Centaurins β1 and β2 show phospholipid regulated GAP activity with high selectivity for ARF6. Confocal fluorescence microscopy of primary cultured keratinocytes transfected with enhanced Green Fluorescent Protein-Centaurin β chimeras indicates that these proteins are difusely distributed in the nucleus and cytoplasm of confluent cells. Dynamic confocal imaging of cells cultured at lower densities and in the presence of growth factors show association of eGFP-centaurin β1 with lamellipodial membranes as well as intracellular circular and tubulovesicular structures. Immunofluorescence confocal microscopy confirms that endogenously expressed β-centaurins associate with the same subcellular structures in cells cultured at low density as well as in keratinocytes at the edge of monolayers following scrape wounding. These studies indicate a possible role for ARF and β centaurin signalling in the regulation of keratinocyte morphological responses to wounding.

0746

The interleukin-6 cytokine system regulates epidermal wound repair in wild-type and IL-6-deficient mice

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Interleukin-6 (IL-6) is a pleiotropic cytokine that is involved in growth and differentiation in numerous cell types and its synthesis is induced by IL-1 and tumour necrosis factor (TNF). In the skin it is produced primarily by epidermal keratinocytes, whereas macrophages, Langerhans cells, and fibroblasts in the dermis represents minor source of the cytokine. Increased levels of IL-6 have been associated with a number of skin pathologies, such as psoriasis and lupus erythematosus. The transcription factor Stat3 is activated by cytokines of the IL-6 family. In the present study we examined the involvement of IL-6, soluble IL-6-receptor, and Stat3 in epidermal wound repair after experimental permeability barrier disruption by tape-stripping. After barrier disruption in wild type mice we found an increased expression of IL-6 and IL-6-receptor in the epidermis using immuno-histochemistry at 0.5 to 5 h after treatment, peaking at 1 and 3 h, respectively. Stat3 tyrosine phosphorylation shown by western blotting was absent at 0.5 h, but induced at 1 h after barrier disruption. In IL-6 deficient mice barrier repair after tape-stripping was significantly reduced at 1 to 24 h after barrier disruption. The effect was reversed by topical application of recombinant IL-6. In wild type mice topical application of IL-6 enhanced permeability barrier repair after wounding. Also, Hyper IL-6, a complex of IL-6 covalently linked to the soluble IL-6 receptor, enhanced barrier repair. Topical application of a soluble form of the signal transducing protein gp130, which has been shown to act as an inhibitor of the agonist IL-6/sIL-6 receptor complex, delayed epidermal wound repair after barrier perturbation. In summary, we found that the IL-6 cytokine system is critically involved in epidermal wound repair. IL-6 may be used to enhance reepithelization in wound healing, whereas, gp130 may be a useful novel therapeutic tool to block IL-6 response in diseased skin.

0747

The epidermal platelet-activating factor receptor augments chemotherapeutic agent-induced apoptosis in human carcinoma cell lines

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Most chemotherapeutic agents exert their cytotoxic effects in part through the induction of apoptosis. In addition, many chemotherapeutic agents are potent pro-oxidative stressors. Although the lipid mediator platelet-activating factor (1-alkyl-2-acetyl glycerophosphocholine; PAF) is synthesized in response to oxidative stress, and many epidermal carcinomas express PAF receptors, it is not known whether PAF is involved in chemotherapeutic agent-induced apoptosis. These studies examined the role of the PAF system in chemotherapy-mediated cytotoxicity using model systems created by retroviral-mediated transduction of the PAF receptor-negative human epidermal carcinoma cell line KB with the human PAF receptor (PAF-R), and ablation of the endogenous PAF-R in the carcinoma cell line HaCaT with a retroviral-mediated tetracycline-inducible antisense PAF-R vector. The presence of the PAF-R in these models resulted in an augmentation of apoptosis (as measured by caspase-3 induction and nucleosomal enrichment) induced by chemotherapeutic agents etoposide and mitomycin C, but not TRAIL or by C2 ceramide. The mechanism by which chemotherapeutic agents activate the epidermal PAF-R involved oxidative stress-induced PAF production, as this process was inhibited by antioxidants, and expression of the fMLP receptor in KB cells did not affect chemotherapeutic agent-induced apoptosis. PAF-R-mediated augmentation of chemotherapeutic agent-induced apoptosis involved the transcription factor nuclear factor (NF)-B because etoposide and mitomycin-C treatment of PAF-R-expressing cells resulted in increased levels of NFkappaB binding and promoter activity. Moreover, NFkappaB pharmacological inhibitors and a super-repressor IkappaBM mutant protein inhibited the PAF-R-mediated augmentation of chemotherapeutic agent-induced cytotoxicity. These studies provide evidence for a novel pathway whereby the epidermal PAF-R can augment chemotherapy-induced cytotoxic effects through an NFkappaB-dependent process.

0748

Identification of a cysteine proteinase from the itch-inducing plant *Macuna pruriens*

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The purpose of this study was to characterize substances from the itch-inducing plant M. pruriens. Isolation of a pure itch-inducing substance could be used as a probe for itch receptors and lead to new therapies for the treatment of pruritus. A putative itch 'proteinase' termed mucanain was described as the active ingredient in the spicules of the seedpods of the cowhage plant M. pruriens by Shelley and Arthur in the 1950s. This proteinase was not characterized further. We have obtained spicules from M. pruriens and extracted material from the spicules under both acidic and alkaline conditions. The extracts have been analyzed using a variety of biochemical approaches including HPLC and gel electrophoresis. The spicules, extracts and the purified material lost activity at basic pH or boiling. Pruritic activity appears to be maintained in the acidic extract and in one of several peaks from size-exclusion HPLC separation. SDS-Page analysis of the total extract and the peaks from the size-exclusion column revealed a major protein band of about 30 kDa in the active fraction. Proteinase activity was present in this fraction. This result suggested that, the active ingredient is not histamine but rather a proteinase. N-terminal and mass spectrometry-based sequencing revealed the presence of a cysteine protease. This proteinase may be a useful tool in determining the mechanism of itch.

0749

Co-operation of ZBP-89 and mitogen-activated protein kinase pathways in the enhancement of matrix metalloproteinase-1 and -3 expression in dermal fibroblasts

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ZBP-89 is a widely expressed four-zinc finger transcription factor that binds to a variety of promoters involved in growth regulation, e.g. type I collagen and stromelysin-1 (matrix metalloproteinase-3, MMP-3). In many instances, ZBP-89 represses the promoter activity by opposing the effect of Sp1. Here, we wanted to study the role of ZBP-89 in the regulation of collagenase (MMP-1) and MMP-3 expression. In dermal fibroblasts, up-regulation of MMP-1 and MMP-3 by tumor necrosis factor-α (TNF-α), but not by interleukin-1β (IL-1β), is enhanced by overexpressing wild-type ZBP-89. Infecting dermal fibroblasts with an adenovirus for constitutively active MKK3b or MKK6b (p38 kinase) specifically activated p38 MAPK and up-regulated the expression of MMP-1 and MMP-3. This up-regulation of MMP-1 and MMP-3 was further enhanced by co-infecting cells with wild-type ZBP-89. Also the up-regulation of proMMP-1 and proMMP-3 production by constitutively active MKK3b and MKK6b was enhanced by co-infection with wild-type, but not dominant negative, ZBP-89. In contrast, the up-regulation of the expression of MMP-1 and MMP-3 by constitutively active MEK (ERK kinase) was not altered by co-infecting cells with wild-type ZBP-89. No effect on type I collagen expression was seen when cells were co-infected with constitutively active MEK1, MKK3b, or MKK6b together with wild-type or dominant negative ZBP-89. These results suggest, that the induction of MMP-1 and MMP-3 expression in dermal fibroblasts by p38 MAPK is mediated through ZBP-89 transcription factor.

0750

Oligonucleotides homologous to the telomeric 3' overhang stimulate p21-dependent adaptive responses to oxidative damage

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We reported that oligonucleotides homologous to the telomeric 3' overhang (T-oligos) induce the same protective responses as sublethal H_2O_2 exposure in human dermal fibroblasts (Fb): Fb treated for 72 hrs with the T-oligo pTT display transient growth arrest, mRNA and protein induction of Cu/Zn- and Mn-dependent superoxide dismutases (SODs), and resistance to oxidative damage as determined by cell yields. We also showed that T-oligo effects are mediated in part by p53 and p21. It is published that ROS participate in signal transduction, via activation of transcription factors or modulation of enzyme activity, and that NADPH oxidases produce ROS in a p53- and p21-dependent manner. We now report that Fb exposure to the T-oligo pGTTAGGGTTAG (40 μM) activates p53, as determined by p53 phosphorylation on serine 15, by 24 and 48 hrs, with a 3-fold higher response than that produced by pTT (100μM) (western blot). A dichlorofluorescein diacetate assay revealed increases in ROS within 36 hrs of treatment with pGTTAGGGTTAG and pTT compared to treatment with control oligonucleotides pCTAACCCTAAC and pCC or diluent alone ($p<0.002$). The specific NADPH oxidase inhibitor diphenyliodonium chloride blocked ROS production and T-oligo failed to induce ROS in p21-null mouse embryonic Fb as compared with wild-type control cells. Within 48 and 72 hrs pGTTAGGGTTAG, but not pTT, modestly induced senescence-associated-B-galactosidase staining, consistent with the ability of prolonged T-oligo treatment ($\propto$ 7 days) to induce Fb senescence. We conclude that in Fb, T-oligo effects depend on degree of homology with the telomeric 3' overhang as well as on length of treatment: only prolonged exposures lead to senescence, while exposures of $\leqq$72 hrs induce ROS levels in a p53- and p21-dependent manner and induce the level of SOD enzymes. These data help elucidate adaptive responses to sublethal oxidative stress mediated by low levels of ROS and the mechanism by which T-oligos mimic these responses in the absence of initial injury.

0751

Study of the involvement of Heregulin and HER family receptors of keratinocytes in epidermal tissue repair

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In human normal epidermis, Heregulin (HRG) and its heterodimeric receptors HER2/HER3 which belong to the human EGF receptor (HER) family constitute a potent autocrine and/or paracrine signaling system. Indeed, HRG is expressed by the non-differentiated keratinocytes of the basal layer, while the receptors (HER2 and HER3) are mainly expressed by the more differentiated keratinocytes of the suprabasal layers. During epidermal wound healing, keratinocytes near the wounded edge alter their phenotype and rapidly initiate cell migration. In this study, we found that keratinocytes of the wounded edge also regulate their expression of HRG and receptors, suggesting a role of this signaling system in the reepithelialization process. In order to test this hypothesis, we analyzed the effect of HRGalpha and HRGbeta on keratinocyte proliferation, differentiation and migration in serum-free culture conditions. Firstly, we found that most HRG isoforms, especially HRGb, enhance the proliferation of low density cultured keratinocytes. Secondly, although we have previously shown that HRGb prevents commitment to keratinocyte differentiation, treatment of already committed differentiating keratinocytes with HRGa or HRGb increases expression of the early differentiation marker keratin 10. These results suggest that HRG may differentially regulate the keratinocyte phenotype. Thirdly, using phagokinetic tracks analysis, we found that HRGb enhances the migration of isolated keratinocytes cultured on collagen I. Conversely, HRGa fails to promote the polymerization of stress fibers. Together, our results suggest that the HRG-HER signaling system in epidermal keratinocytes is involved in the control of the reepithelialization process, apparently acting on both the control of the proliferation-differentiation balance and the migration process of keratinocytes.

0752

4-tert-butylphenol and UVB stimulate distinct apoptotic pathways in human melanocytes

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In previous studies we found that human melanocytes express a broad spectrum of apoptosis inhibitors and are relatively resistant to most apoptotic stimuli, with the exception of the tyrosine analog 4-tert-butylphenol (4-TBP). To investigate the mechanistic basis for melanocyte susceptibility to 4-TBP-induced apoptosis, we examined apoptotic events and pathways in melanocytes optimally stimulated with 0.9 mM 4-TBP or 1200 J/m^2 UVB. For both stimuli, DNA fragmentation (assessed by propidium iodide staining and flow cytometry) was detected by 24 hrs and was maximal by 48 hrs, although the magnitude was far greater in cells treated with 4-TBP compared to UVB. There was activation of upstream caspase-9 and terminal caspase-3 (as assessed by precursor loss and appearance of cleavage fragments on Western blots) in both 4-TBP and UVB-treated cells, but activation of upstream caspase-8 was seen only after UVB treatment. Apoptosis induced by both stimuli was partially (50%) blocked by the pan-caspase inhibitor ZVAD. Interestingly, cells treated with 4-TBP after 1 hr were non-adherent, and there was complete transposition of phosphatidylserine (determined by PE-Annexin V binding). Mitochondrial depolarization (JC-1 fluorescence shift) and release of cytochrome c and Smac/DIABLO (Western blot) were also seen in 4-TBP-treated melanocytes after only 1 hr. By contrast, melanocytes exposed to UVB generally remained adherent and did not exhibit Annexin V binding until 24-48 hrs later. Similarly, mitochondrial depolarization and content release did not occur in UVB-treated cells until after 24-48 hrs. These results suggest that despite similar kinetics and partial caspase-independence of DNA fragmentation induced by 4-TBP and UVB, distinct apoptotic pathways are stimulated. The susceptibility of melanocytes to 4-TBP-induced apoptosis likely relates to the capacity of this compound, unlike UVB, to cause rapid cell detachment, phosphatidylserine transposition and mitochondrial apoptotic events.

0753

Serotoninergic and melatoninergic systems expressed in the skin

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We have shown in mammalian skin expression of genes for tryptophan hydroxylase, arylalkylamine N-acetyltransferase and hydroxyindole-O-methyltransferase together with the corresponding enzymatic activities (FASEB J 10.1096/fj.0.1-0952fje; J Invest Dermatol 119,934-942). The enzymes are arranged into pathways since serotoninergic and melatoninergic products were also detected, including serotonin (5HT), N-acetylserotonin (NAS), melatonin (MT) and 5 methoxytryptamine (5 MTT) in human skin and selected cultured skin cells (FEBS Lett 511,102-106). Currently we are showing expression of genes coding receptors of a cutaneous serotoninergic/melatoninergic system in whole human skin and in normal and pathologic cultured skin cells. Evaluation of 5HT, MT and MT-related receptors (MRR) showed expression of the isoforms 5HT2B, 5HT7 and MT1 genes in almost all the tested samples. Expression of other isoforms was less prevalent; 5HT2C, MRR and MT2 were rarely detected. We also found novel isoforms for MT2, MRR and 5HT2B, and document RNA editing for 5HT2C. Testing for functional activity of these receptors with 5HT and MT (10-14 to 10-10 M) showed variable effects depending on cell type and culture conditions. Thus, 5HT stimulated proliferation of melanocytes in medium deprived of growth factors, while inhibiting cell growth in the presence of growth factors. MT inhibiting apoptosis of HaCaT keratinocytes in serum-free media, it also inhibited proliferation of cells cultured in medium supplemented with serum. MT also increased fibroblasts survival in serum free medium. NAS and 5MTT were generally without effect on cell proliferation. Thus, skin cells represent a target for the local serotoninergic/melatoninergic pathway.

0754

Delineating an insulin specific signaling pathway in skin proliferation: the role of protein kinase C delta and transcriptional activation of STAT3

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In our previous studies we identified a unique signaling pathway, which specifically mediates insulin signaling in skin keratinocyte proliferation and involves PKCδ activation. In the present study we have identified the role of the transcription factor STAT3 (Signal Transducers and Activators of Transcription) in this pathway utilizing a model of skin keratinocytes in culture. STAT3 activation is a multi-step process associated with STAT3 phosphorylation, dimerization, followed by nuclear translocation and binding to target genes to induce transcription. It was found that in keratinocytes, PKCδ but not other PKC isoforms expressed in skin is constitutivly associated with STAT3. Following insulin stimulation, serine and tyrosine phosphorylation of STAT3 was induced followed by STAT3 nuclear translocation and transcriptional activation. Specifically, overexpression of PKCδ induced STAT3 serine phosphorylation and its association with PKCδ. Inhibition of PKCδ activity by overexpressing a PKCδ mutant, or pharmacological inhibition by rottlerin, abrogated insulin induced activation of STAT3 and blocked STAT3 mediated proliferation. Moreover, overexpression of a STAT3 tyrosine mutant, inhibited insulin induced PKCδ activation and its association with STAT3. Finally, In order to explore the significance of STAT3 serine phosphorylation to insulin signaling, a STAT3 serine mutant adenovirus was constructed. As expected, overexpression of STAT3 serine mutant abrogated insulin induced STAT3 serine phosphorylation. However, in cells overexpressing the STAT3 serine mutant STAT3 activation was dramatically increased several folds above control levels. Overall, these results suggest a physical and functional link between PKCδ and serine phosphorylation and activation of STAT3 in mediation of insulin-induced proliferation in skin keratinocytes.

0755

Role of PKCδ and STAT3 activation in insulin signaling in skin keratinocytes

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Insulin belongs to a family of insulin like growth factors associated with diverse biological actions. Binding of insulin to the insulin receptor induces tyrosine phosphorylation and activation of the receptor leading to recruitment and phosphorylation of the insulin receptor substrate (IRS) and initiation of the signaling cascade. Following phosphorylation and activation, IR and IRS become platforms for other signaling molecules downstream of the Insulin signaling pathway. We have previously identified a novel pathway in keratinocyte proliferation which involves insulin induced activation of PKCδ. Utilizing mouse primary keratinocytes we have further characterized the signaling cascade associated with insulin stimulation of keratinocytes. In Insulin treated cells, IR and IRS1 are tyrosine phosphorylated and activated in a dose and time dependent manner. In response to insulin stimulation PKCδ physically associated with IR, but not with IRS1 or IRS2. Interestingly, overexpressing PKCδ induced maximal IR phosphorylation regardless of insulin stimulation. Furthermore, overexpression of a dominant negative form of PKCδ abolished insulin induced tyrosine phosphorylation of IR. Insulin also induced PKCδ association with Signal Transducer and Activator of Transcription (Stat3). As early as 5 minutes following insulin stimulation, Stat3 phosphorylation and association with PKCδ was induced. Overexpressing wild type Stat3 or inhibiting Stat3 by overexpressing a dominant negative tyrosine mutant did not alter IR tyrosine phosphorylation in response to Insulin. In contrast, overexpression of a Stat3 serine mutant induced constitutive phosphorylation of IR, similarly to overexpression of PKCδ. In conclusion: PKCδ and Stat3 are down stream effectors of the insulin-signaling pathway in skin keratinocytes. The ability of PKCδ and Stat3 to affect phosphorylation of the IR suggests a role for PKCδ and Stat3 in upstream insulin signaling in skin keratinocytes.

0756

Ultraviolet irradiation induces epidermal growth factor receptor nuclear translocation in human keratinocytes

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Epidermal growth factor receptor (EGFR) plays a critical role in mediating ultraviolet (UV) irradiation-induced signal transduction and gene expression in human keratinocytes (KC). EGFR activation results from increased phosphorylation on specific tyrosine residues in the C-terminal intracellular domain. It has recently been reported that upon growth factor stimulation EGFR translocates from the surface membrane to the nucleus, where it directly regulated gene transcription. We have investigated the ability of UV irradiation to induce EGFR nuclear translocation in human KC. UV irradiation (40mJ/cm^2, UVB/A2) caused rapid nuclear translocation of EGFR. Significant accumulation of EGFR in the nucleus was observed within 5 minutes of UV exposure. Maximal translocation occurred at 30 minutes post UV, and resulted in a 10-fold increase (n=5) in EGFR in the nucleus, as determined by Western analysis of isolated nuclear extracts. Epidermal growth factor (EGF) also caused EGFR nuclear translocation, but to a lesser extent (3-fold, n=5) than UV. Nuclear localization of EGFR following UV irradiation or EGF was confirmed by immunofluorescence. Leptomycin B (nuclear export inhibitor) did not alter UV-induced nuclear accumulation, indicating that the mechanism of nuclear localization did not involve reduced nuclear export. However, inhibition of UV-induced EGFR tyrosine phosphorylation by the specific inhibitor PD169540 reduced nuclear translocation 70%. To further explore the role of EGFR tyrosine phosphorylation, the effect of point mutation of specific tyrosine residues on UV-induced EGFR nuclear translocation was examined. Mutation of either tyrosine 1148 or tyrosine 1173 reduced nuclear translocation 70%, while mutation of tyrosine 1068 was without effect. These data demonstrate that UV irradiation stimulates rapid EGFR nuclear translocation, which is dependent on phosphorylation of specific tyrosine residues. EGFR nuclear translocation is a heretofore-unrecognized signaling pathway mediating UV-induced responses in human KC.

0757

The role of JAK3 and STAT3 in mastocytosis

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The tyrosine kinase receptor KIT is essential for the growth and development of mast cells. Studies have shown that a point mutation (816) in c-kit leads to tyrosine kinase autoactivation which has been proposed as the cause of adult-onset mastocytosis. One strategy for treating mastocytosis focuses on inhibiting the downstream KIT signaling pathways JAK3/STATs. Previous studies form our laboratory have shown that the JAK3/STAT inhibitor Whi-p131 reduced the growth of two human mastocytosis mast cell lines (HMC-1 and RMT) by 82±14% and 87±8% respectively. To study further the functional role of the JAK3/STATs in mastocytosis mast cell growth, cellular extracts were analyzed for the presence of activated JAK3 and STATs by Western blots. While both cell lines expressed significant amounts of JAK3, only low levels of STAT1, STAT5 and STAT6 were detected. However, both HMC-1 and RMT expressed high levels of the anti-apototic, phosphorylated STAT3 protein (ser727). Treatment of these cell lines with Whi-p131 for 4 hours failed to reduce the expression of either JAK3 or phosphorylated STAT3 despite the fact that this inhibitor greatly reduced the growth of both mastocytosis cell lines. We conclude from these studies that Whi-p131 has the ability to significantly reduce the growth of mastocytosis mast cells; however, its mechanism of action is yet to be defined. Nevertheless, this inhibitor may prove to be useful for the treatment of cutaneous lesions of adult mastocytosis patients.

0758

Microarray analysis of temporal changes in gene expression induced by TGFβ1 in the mouse skin

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Transforming growth factor beta 1 (TGFβ1) is an important regulator of epidermal growth and homeostasis. Elevated expression of this cytokine occurs frequently in a variety of pathological conditions including cancer, in the skin and other epithelial tissues. We have utilized cDNA microarray analysis in conjunction with a conditional expression model to examine patterns of TGFβ1-induced gene expression in the intact normal epidermis. In this model active TGFβ1 is induced in the epidermis of mice transgenic for both a keratin 5 promoter driven rTA and a tetOTGFβ1 cassette by the addition of doxycycline. RT-PCR analysis of RNA isolated from whole skin shows that within 24 hrs of addition of doxycycline, TGFβ1 transgene mRNA is induced to near maximal levels. We have used NCI mouse cDNA microarray slides containing 10,000 features to examine the transcriptional response to elevated TGFβ1 at specific times after transgene induction. At each time point RNA was isolated from the skin of 4 double and single transgenic mice from which the underlying muscle and fascia had been removed. At least 4 comparisons from independent samples were done for each time point. We observed 3 major patterns of gene expression in response to TGFβ1: genes that were rapidly but transiently induced or repressed at 24 hrs, genes whose induction or repression was sustained over multiple time points, and genes with a delayed induction pattern. As expected known TGFβ1 responsive genes were identified within the differentially regulated gene set. In addition, epidermal differentiation markers such as keratins 3, 4 and 10, filaggrin and sprr were rapidly downregulated while muscle differentiation markers were induced. There were significant changes in genes involved in cell adhesion, gap junction formation, and extracellular matrix. These results point to complex temporal patterns of gene expression in response to TGFβ1 *in vivo* and suggest that this type of analysis will provide important information on the mechanism of TGFβ1 based pathologies.

0759

IL-7-diphtheria toxin fusion. DNA construction and expression

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Constitutive expression of the IL-7 receptor is a prominent characteristic of cutaneous T cell lymphoma (CTCL), and it is detected in one third of the NCI-60 panel of tumor cell lines. This makes the receptor an attractive target for fusion toxin therapy of cancer cells. Fusion toxins consist of a high affinity ligand for a target cell surface receptors and toxin such as the A and B domains of diphtheria toxin (D_{AB}). D_{AB}-IL2 selectively kills cells expressing the high affinity IL-2 receptor and is used clinically for CTCL. A parallel toxin directed against the IL-7 receptor, D_{AB}-IL7, has been made in bacteria but it has been difficult to produce in sufficient quantity and quality to develop. A likely reason for this is that bacterial methods for producing the toxin rely on denaturation and refolding: IL-7 has 6 cysteines, which form 3 disulfides, while IL-2 has only 4 cysteines, which form 2 disulfides. To circumvent this we are attempting to engineer in mammalian cells to secrete active D_{AB}-IL7. We have isolated variants of several cell lines that are resistant to high concentrations of diphtheria toxin and assembled expression constructs encoding D_{AB}-IL7 and D_{AB}-GFP that are suitable for mammalian cells. Diphtheria toxin resistant cells transfected with D_{AB}-GFP accumulate GFP while toxin sensitive cells do not, demonstrating that diphtheria toxin can be made in mammalian cells. We believe that this will prove efficient system for the production of active D_{AB}-IL7, allowing it to be tested in animal models of lymphoma. Characterization of D_{AB}-IL7 produced in this system is ongoing.

0760

Involvement of adiponectin in the cutaneous wound healing process

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Adipose tissue secretes complement factors, growth factors and cytokines and participates in self-defense responses. Adiponectin is a secretory protein exclusively produced by adipocytes. Here, we investigated the role of adiponectin in cutaneous wound healing. In humans, adiponectin was overexpressed in adipose tissue beneath the epithelial tongue of acute wounds. Mice lacking adiponectin showed retardation of the wound healing process characterized by poor reepithelialization and increased granulation tissue, compared with the wound of wild type littermates. In cultured keratinocytes, adiponectin enhanced the phosphorylation of ERK1/2 and promoted proliferation and migration of keratinocytes in a dose-dependent manner. Our results suggest adiponectin participates in the normal wound healing process.

0761

Conditionally active Raf1 and MEK1 inducibly alter epidermal growth and differentiation in transgenic mice

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A number of pathways exert important effects on epithelial growth and differentiation, including the Ras/Raf/MEK/MAPK cascade. Recent work indicates that activation of oncogenic Ras in epidermis promotes proliferation and opposes terminal differentiation, however, the role of the three major specific Ras effector pathways, Raf/MEK/ERK, PI3K/Akt and RalGEF/Ral has not been defined. To address this, we generated mice with conditionally active Raf1 and MEK1 in epidermis via 4-hydroxytamoxifen (4OHT)-responsive C-terminal estrogen receptor fusions with Raf1 and MEK1 kinases (K14-Raf:ER and K14-MEK1:ER mice, respectively). Induction of Raf1 and MEK1 activity in response to topical 4OHT was confirmed by enhanced phosphorylation of their downstream target MAP kinase (MAPKs ERK1 and ERK2). Epidermal hyperplasia and inhibited expression of some differentiation markers was observed with induction of both kinases. These changes resembled those seen with Ras induction and indicate that Raf/MEK activation is sufficient to effect these Ras-driven epidermal alterations. We next examined the necessity of specific members of the Raf/MEK cascade for Ras action. Conditionally inducible epidermal Ras mice (K14-ER:Ras) were crossed with MEK2 knock-out mice to test if MEK2 is required for Ras effects on epidermal growth and differentiation. Induction of Ras in K14-ER:Ras/MEK2-/- mice led to proliferation and inhibited differentiation that was indistinguishable in kinetics and magnitude to K14-ER:Ras/MEK2+/+ controls, indicating that MEK2 is dispensable for epidermal changes driven by oncogenic Ras. These data indicate that induction of the Raf/MEK/ERK effector cascade is sufficient to mediate epidermal Ras effects on growth and differentiation and suggest that MEK1 and MEK2 are redundant in this setting.

0762

SIRKO-the skin specific insulin receptor knockout mouse

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Impaired wound healing in diabetes is associated with high morbidity and mortality rates. However the pathogenesis leading to this complication is not known. Previously we have demonstrated direct involvement of insulin in regulation of normal skin physiology and shown that lack of insulin receptor (IR) expression leads to abnormal skin proliferation and differentiation. However direct investigation of the involvement of the IR in wound healing in vivo was not possible since the IR null mice available die within 5-7 days after birth. To resolve this problem we developed the epidermal skin-specific insulin receptor knockout mouse (SIRKO) driven by matings of IRflox and K5Cre mice. The SIRKO mice exhibit an epidermal-specific reduction (> 95%) in receptor content and early signaling events. This was associated with changes of the epidermis that appeared flatter and thinner compared to the wild type skin. Similarly to the skin cells of the IR whole body knockout mouse, the SIRKO skin cells exhibited impaired differentiation both in vivo and in vitro. However the striking change was seen in the dermis of the SIRKO mice. While there was no change in the IR content in the dermal fibroblasts, there was a progressive thickening of the dermis due to increase in closely packed collagen bundles and the disappearance of the subdermal adipose tissue. In wound-healing studies being conducted, deterioration in the strength of the healed wound is detected. These results suggest that insulin signaling in skin keratinocytes is essential for normal skin function and furthermore, impairment in its function might contribute to the impaired wound healing process characteristic to diabetes.

0763

Downstream signaling pathways that mediate protein kinase C-dependent keratinocyte cell death—a novel p38δ/ERK1/2 complex

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It is thought that several protein kinase c (PKC) isoforms function as master controllers regulating the processes that determine keratinocyte survival, death and differentiation. As such these kinases are pivotal regulators of keratinocyte function. Despite this realization, the downstream signaling pathways that mediate this action have not been clearly defined. Our present studies implicate several mitogen-activated protein kinases (MAPKs) as downstream carriers of the death signal. Expression of PKCδ in keratinocytes causes limited cell death; however, co-expression with p38δ MAPK produces profound alterations in cell appearance, including nuclear shrinkage, cell rounding, cell detachment, and enhanced cornified envelope formation. Annexin V/propidium iodide staining and Hoescht staining confirm induction of apoptosis. PARP degradation and procaspase-3 cleavage is also observed. This highly reproducible response is absolutely specific for the PKCδ/p38δ combination, and is not observed when p38δ is replaced by p38α, or other PKC isoforms are substituted for PKCδ. At the MAPK kinase level, this response is reproduced by co-expression of MEK6 with p38δ, and not when MEK3 or MEK1 are substituted for MEK6. Moreover, a reduction in ERK1/2 activity appears to be an integral requirement for cell death, since PKCδ/p38δ co-expression reduces ERK1/2 activity, and expression of constitutively-active Raf-1, which reactivates ERK1/2, prevents cell death. These findings suggest that a PKCδ triggers keratinocyte death by activating MEK6 which, in turn, activates p38δ. We further provide evidence that a p38δ and ERK1/2 exist as a complex and that p38δ may directly influence ERK1/2 activity. These results indicate that PKCδ triggers cell death by tilting the balance of p38δ and ERK1/2 activity in favor of p38δ.

0764

Endogenous TGF-β and endothelin differentiate human skin fibroblasts into myofibroblasts in keratinocyte co-culture: counter-regulation by endogenous IL-1

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Epithelial-mesenchymal interactions are crucial to skin development, homeostasis and wound healing. In the latter, dermal fibroblast (DF) derived growth factors are know to contribute significantly to the regulation of keratinocyte behaviour, yet the influence of keratinocytes on DF metabolism remains largely unknown. We have used several co-culture and read-out systems to systematically investigate the influence of keratinocytes on DFs: cDNA microarray screening yielded over 100 genes differentially regulated in DFs upon co-culture with keratinocytes. Numerous extracellular matrix and smooth muscle cell associated genes were strongly up-regulated, suggesting a differentiation into myofibroblasts (MF). We confirmed this by analyzing the expression of the MF marker, α-smooth muscle actin (SMA). MF differentiation required time and close proximity between keratinocytes and DFs. Exogenous growth factors were dispensable, however, MF differentiation required both endogenous TGF-β and endothelin activity: TGF-β neutralizing mAb and an endothelin receptor antagonist abolished SMA induction. Accordingly, TGF-β production and activation were increased in co-culture, as were endothelin 1 protein and mRNA transcripts in co-cultured DFs. Blocking keratinocyte derived IL-1 with IL-1ra, we could show an unexpected SMA induction in co-cultures. The NFkB binding activity is increased in co-cultured DFs, possibly inhibiting TGF-β mediated transcription. Indeed, addition of exogenous IL-1 abrogates SMA induction in co-cultured DFs and in DFs stimulated with TGF-β1. In summary, co-culture with keratinocytes effectively differentiates DFs into MFs via a complex interplay of positively and negatively acting factors.

0765

Requirement of Akt/PKB for PDGF-BB-, but not collagen-, induced migration of human dermal fibroblasts

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Extracellular matrices (ECMs) and growth factors (GFs) are the two main extracellular cues that directly influence the decision of a cell to move or stop. The optimal migration of human dermal fibroblasts (HDFs) is driven by a collagen matrix and platelet-derived growth factor-BB (PDGF-BB) in vitro. To understand the mechanisms of collagen and PDGF-BB signaling, we investigated the role of Akt/PKB, a prominent downstream target for the PI-3K pathway. Both PDGF receptor and integrin activate PI-3K. We found that PDGF-BB, but not collagen stimulation potently activated Akt in HDFs as detected by anti-phospho-Akt antibodies. Using lentiviral infection to achieve over 95% gene transduction efficiency in primary HDFs, we studied if Akt is required for collagen and PDGF-BB-stimulated HDF migration. The cells were infected with lentivirus carrying either a dominant negative (Akt-K179M) or a constitutively active (Myr-Akt) mutant of human Akt1. Western blot analysis showed that the level of the exogenously expressed Akt was 5-7 fold higher than that of endogenous Akt (including Akt1, 2, 3). The results showed that the dominant negative mutant, but not the wild type Akt, completely blocked PDGF-BB-stimulated migration, whereas it had little effect on collagen initiation of migration. Activation of Akt appeared to be necessary but not sufficient for PDGF-BB to stimulate HDF migration since overexpression of the constitutively active Myr-Akt had no significant stimulatory effect. Furthermore, while participation of all three major mitogen-activated protein kinase cascades, ERK1/2, p38 and JNK, were implicated in PDGF-stimulated HDF migration, blockade of Akt signaling selectively inhibited the PDGF-BB-stimulated p38 activation. This suggests that the ERK1/2 and JNK pathways act in parallel with the Akt-p38 pathway to mediate the PDGF-BB signaling. In conclusion, this study elucidates the role of Akt and its mechanism of action in PDGF-BB-stimulated migration of HDFs.

0766

Focal adhesion kinase but not Src plays an important role in human keratinocyte migration

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Signals from extracellular matrices (ECMs) and serum growth factors (GFs) are the two main extracellular cues that control human keratinocyte (HK) migration. We previously showed that ECMs, but not GFs, can initiate migration. However, along the ECM-induced migration is partial and non-directional. We hypothesized that the interaction of HKs with ECM is permissive to migration. The ECMs signals then work in concert with GFs to optimize motility. In this study, we further investigated these dual signaling pathways. We focused upon focal adhesion kinase (FAK) and pp60Src, two protein tyrosine kinases known to be important for integrin signaling and also linked to the growth factor receptor signaling. We found that FAK and Src are rapidly activated in HKs migrating on collagen in the presence of GFs. As expected, blockade of FAK signaling by FAK dominant negative mutants (R454, F397Y and FRNK) significantly decreased tyrosine phosphorylation of Src. However, over expression of kinase-inactive Src had little effect on the activation of FAK in the same cells. To study the importance of FAK and Src in HK migration, we inhibited either FAK or Src signaling by infecting cells with lentiviral vectors containing the three FAK mutants or a kinase-inactive Src mutant. The cells were then subjected to a colloidal gold migration assay on a collagen matrix. We found that all three FAK mutants, but not their wild type (wt) counterpart, potently blocked both collagen-initiated and GF-stimulated HK migration. In contrast, neither the wt nor the kinase-inactivated mutant of Src had any significant effect. Taken together, this is the first report that directly addresses the roles of FAK and Src in HK migration. We conclude that FAK and Src play distinct roles.

0767

E-cadherin can inhibit activation of receptor tyrosine kinases and transformation by oncogenes

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The purpose of this study was to identify mechanisms by which the tumor suppressor gene E-cadherin, which is required for normal development and is frequently down-regulated in epithelial tumors and melanomas, negatively regulates cell growth. We here present evidence that endogenous E-cadherin can inhibit mitogenic signaling from receptor tyrosine kinases (RTK), including EGFR, IGF1R, and Met, by impairing the response of RTKs to their cognate ligands. E-cadherin was found to form stable complexes with RTKs. In confluent cells, complex formation was associated with reduced RTK mobility within the plasma membrane, as determined by FRAP (fluorescence recovery after photobleaching), and lowered the efficiency of ligand-induced RTK dimerization and activation. These effects, which can be abrogated by an E-cadherin function blocking antibody, appear to be specific for RTKs, as E-cadherin did not inhibit signaling via G-protein coupled receptors. Results from colon cancer cell lines carried out by other laboratories have attributed most of the growth inhibitory activity of E-cadherin to the ability of its C-terminus to bind and sequester beta-catenin. However, we have found with several other cell types (kidney epithelial cells, melanocytes, and fibroblasts) that E-cadherin can form complexes with RTKs and inhibit RTK signaling even without beta-catenin binding, since these functions are retained by E-cadherin premature termination mutants that are deficient for binding beta-catenin. In these cell types, E-cadherin wild type and premature termination mutants can inhibit cell transformation by ras and src oncogenes, presumably because full transformation depends on the activation of RTK pathways via serum factors and autocrine factors. Thus, the ability of E-cadherin to suppress RTK signaling has implications for normal and abnormal cell growth.

0768

Lysophosphatidic acid-induced EGF receptor internalization in human keratinocytes depends on metalloproteinase activity

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Several recent reports showed that transactivation of the ErbB1 by G protein-coupled receptor (GPCR) ligands requires metalloproteinase (MP) activity in several cell types. However, this process has not previously been studied in normal human keratinocytes (NHK). Therefore, we asked whether the GPCR ligands lysophosphatidic acid (LPA) and carbachol could also induce transactivation of ErbB1 in NHK, and whether this process could be blocked by inhibitors of MP activity. NHK were grown until 40-50% confluent followed by incubation in growth factor-free basal medium for 48 h. Cells were then treated with LPA, carbachol or EGF for various times in the presence or absence of the ErbB1 receptor tyrosine kinase inhibitor (RTKI) PD158780 (1 uM) or the MP inhibitor BB2516 (20 uM). After fixation, NHK were stained with anti-ErbB1 antibodies and analyzed by immunofluorescence microscopy. ErbB1 showed a well-defined cell surface staining in untreated NHK. Treatments with EGF (10 min) or LPA (15 min) rapidly led to the internalization of ErbB1 as evidenced by the development of punctate intracellular staining. Preincubation of NHK with ErbB RTKI PD158780 blocked both EGF- and LPA-induced internalization, whereas the MP inhibitor BB2516 blocked LPA-induced, but had no effect on EGF-induced internalization. In contrast to LPA, an alternative GPCR ligand carbachol had no effect on ErbB1 internalization in NHK. Consistent with rapid degradation of internalized receptors, we have consistently observed a marked (30-50%) decrease of ErbB1 protein in NHK after 10 min of EGF stimulation. This decrease can be blocked by pretreatment of the cells with PD158780. These data indicate that metalloproteinase-dependent transactivation of ErbB1 by selective GPCR ligands such as LPA leads to rapid internalization of ErbB1.

0769

p38 mitogen-activated protein kinases (MAPKs) mediate ultraviolet B (UVB)-induced COX-2 expression and inflammation in the skin of SKH-1 hairless mice

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MAPKs are a family of serine/threonine protein kinases including extracellular signal-regulated protein kinases (ERK), p38 MAP kinase (p38), and c-Jun NH2-terminal kinases (JNK). Signaling pathways involving MAP kinases respond to various cytotoxins and environmental stresses including solar radiation. UVB is a potent environmental carcinogen and a known risk factor for human non-melanoma skin cancers (NMSC). UVB increases p38 activities, and a p38 inhibitor abrogates UVB-induced COX-2 expression in human epidermal keratinocytes. Recently, we showed that COX-2 is up-regulated during UVB-induced skin carcinogenesis in SKH-1 hairless mice. In this study we assessed the effects of UVB on the p38-MAP kinase signaling pathway and determined whether COX-2 over-expression in the skin of UVB-irradiated SKH-1 mice is downstream of this kinase. SKH-1 mice were irradiated with a single erythema dose of UVB (360 mJ/cm2), and epidermal homogenates evaluated for p38 MAP kinase activity. UVB induced phosphorylation of p38 and its upstream kinases MKK3/6 in a time-dependent manner. Phosphorylation of MAPK activated protein kinase 2 (MAPKAPK-2) was also detected at both Thr222 and Thr334 residues. This increase in phospho-MAPKAPK-2 correlated with an increase in kinase activity. MAPKAPK-2 activation also resulted in phosphorylation of heat shock protein (hsp27), an endogenous substrate. UVB-induced activation of the p38 MAPK cascade was inhibited by pre-treatment with a single dose of SB-242235, a known inhibitor of this kinase prior to UVB irradiation. SB-242235 abolished MAPKAPK-2 kinase activity as well as phosphorylation of hsp27. UVB-induced COX-2 over-expression was simultaneously inhibited as were the pro-inflammatory cytokines IL-6 and IL-8 by SB-242235 treatment. Our data demonstrate that UVB activates epidermal p38 MAPK and COX-2 over-expression. Inhibitors of p38 may offer a novel approach to cancer chemoprevention by diminishing UVB-induced inflammation and modulating tumor promotion.

0770

Active Ras upregulates pAkt and increases anti-apoptotic defenses via a PI3K-specific mechanism

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We investigated the effects of activating Ras on the autonomous anti-apoptotic defenses in melanoma that are mediated by PI3K. PI3K phosphorylates Akt (pAkt), which has multiple downstream targets that support cell survival. It was our hypothesis that in melanoma, activating Ras maintains anti-apoptotic defenses by activating the PI3K pathway, and through pAkt, leads to increased activity of the transcription factor, CREB, resulting in upregulation of Bcl-2 and potentially other anti-apoptotic regulatory mechanisms. We used the following cell lines to investigate our hypothesis: WM35H-Ras and WM35 N-Ras (containing a constitutively active mutant H-Ras or N-Ras oncogene, respectively) and WM35Neo (control). Previously, we have found that transfecting WM35 cells with an activating Ras oncogene leads to increased malignancy and confers survival advantages: resistance to cell death from Fas, cisplatinum, and UVR. For our experiments, the cells were grown as spheroid cultures thereby eliminating any survival signals due to integrin binding to tissue culture plastic. In the absence of serum, there was no difference in the level of pAkt (Western Blot) between the Ras transfectant lines and the control, and the level of pAkt was quite low. However, when stimulated with 10% FBS, pAkt increased 3-fold in WM35Neo, 4-fold in WM35N-Ras, and 6-fold in WM35 H-Ras. Treating these cells with the PI3K inhibitor, LY294002, at 500uM for 24 hrs decreased pAkt to just above baseline. In addition, Bcl-2 was significantly decreased in all three cell lines treated with PI3K inhibitor compared to serum stimulation alone. The melanoma cell line, WM35, when grown in spheroid culture is dependent upon serum stimulation for pAkt expression. This effect is augmented approximately two-fold by an activating H-Ras mutation. It appears that activating Ras activity contributes to melanoma progression, at least in part, by stimulating the PI3K pathway, resulting in pAkt-induced upregulation of anti-apoptotic defenses, including Bcl-2, in the melanoma cell line, WM35.

0771

Homeobox factors HOXA7 and HOXA5 exhibit differential regulation of keratinocyte growth, differentiation, and apoptosis

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Homeobox transcription factors regulate differentiation and cell fate during development, and in tissues undergoing continuous renewal through proliferation and differentiation. We have reported that HOXA7 inhibits differentiation in transfected primary human keratinocytes. Paralogous members of the Class 1 homoeobox (HOX) transcrition factor family, as well as the products of neighboring HOX genes, appear to share overlapping, and perhaps synergistic function. In addition, both HOXA7 and HOXA5 exhibit downregulation in keratinocytes induced to differentiate. We therefore have initiated a comparison of HOXA7 and HOXA5 function in human keratinocytes. A set of adenoviral vectors was prepared containing the HOXA7 and HOXA5 full-length cDNAs, or truncated 5' cDNA fragments lacking code for the homeobox DNA-binding region, each in the forward and reverse orientations. The viral vectors contain tandem cytomegalovirus promoter-driven cassettes for simultaneous eGFP and HOX expression, allowing viral titration by GFP fluorescence. As seen previously in transiently-transfected keratinocytes, adenoviral HOXA7 expression inhibited transglutaminase type 1 gene transcription, as a marker of differentiation, and antisense HOXA7 expression resulted in stimulation of transglutaminase 1 expression. Interestingly, exogenous HOXA5 expression also downregulated the transglutaminase type 1 gene. Both HOXA7 and HOXA5 overexpression triggered an increased rate of apoptosis absent from cells harboring the antisense constructs. This activation of cell death appeared to require the DNA-binding homeodomain. However, HOXA7-transduced cultures nevertheless exhibited increased cell numbers. Antisense HOXA5 expression stimulated proliferation, an effect not seen with antisense HOXA7 expression. These results suggest that in undifferentiated human keratinocytes, both HOXA7 and HOXA5 are potential inhibitors of differentiation, but that endogenous HOXA7 suppresses differentiation, while endogeous HOXA5 acts to limit cell growth.

0772

Srcasm activates src-family tyrosine kinases *in vivo* and modulates EGF-dependent signaling pathways

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Srcasm: Src activating and signaling molecule, is a substrate and in vitro activator of Src-family tyrosine kinases (SFKs). In this study, we demonstrate that Srcasm activates Fyn, a Src-family kinase, in vivo and modulates the activation of molecules associated with EGF receptor signaling. Co-transfection of COS cells with Fyn and increasing amounts of Srcasm results in correspondingly higher levels of Fyn phosphorylated at tyrosine 416, which indicates the amount of activated kinase. To examine the effects of Srcasm on SFK regulated signaling pathways, COS cells were tranfected with Srcasm and the levels of tyrosine phosphorylated EGF receptor were determined by western blotting. In quiescent and EGF stimulated cells, Srcasm promoted EGF receptor tyrosine phosphorylation. In addition, immunofluorescence studies demonstrate that Srcasm co-localizes with Fyn and EGF receptor. The ability of Srcasm to promote signaling events downstream of the EGF receptor was evaluated by performing serum-response element dependent luciferase transcriptional assays. Native Srcasm can potentiate the ability of Fyn to stimulate transcription from serum-response elements while mutated forms of Srcasm cannot. Initial biological studies reveal that Srcasm promotes cellular proliferation in A431 cells in an EGF dependent and independent manner. These studies demonstrate that Srcasm can activate Src-family kinases and promote signaling in SFK-dependent pathways. Together, this information suggests that Srcasm may play a role in regulating cellular proliferation.

0773

Transgenic mice overexpressing the calcium sensing receptor express aberrant epidermal differentiation

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The regulation of growth and differentiation by calcium (Ca++) is fundamental in cell physiology. However despite much progress in elucidating Ca++ sensitive signalling pathways, many details coupling changes in Ca++ to biological or developmental outcomes are missing. Epidermal cells are one cell type in which Ca++ exerts a marked effect on growth and differentiation, however it is not understood how the cells "feel" the changes in Ca++ and initiate the differentiation process. The recent identification of the calcium sensing receptor (CaSR), a member of the G protein-coupled receptor family led us to ask what role it might play in epidermal differentiation. We therefore engineered transgenic mice with the CaSR overexpressed via the keratin 14-(K14) promoter, which drives expression in the basal layer. The transgenic mice displayed abnormalities in epidermal differentiation with strikingly accelerated hair growth. Changes in differentiation were clearly manifested biochemically as detected by aberrant expression of late epidermal differentiation markers, including K1, filaggrin, loricrin, transglutaminase-3, and involucrin. These results provide new insights into the role of CaSR in epithelial differentiation and hair formation. This work was supported by grant from NSERC of Canada.

0774

BEG4 is a novel actin binding protein expressed in hair follicles and BCCs

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The Shh signaling pathway plays a critical role in the development of multipotent progenitor cells, and inappropriate induction of its target genes has been implicated in the development of BCCs of the skin. However little is known about how Shh target genes mediate the morphogenesis associated with development and tumorigenesis. BEG4 is a novel Shh target gene identified through cDNA microarray screen of human BCCs. BEG4 is a proline-rich protein with a coiled-coil N-terminal and with a C-terminal domain that shares a conserved WH2 domain with the human Wiskott-Aldrich Syndrome protein (WASP). WH2 domains are highly conserved motifs that bind to G-actin and act downstream of the small Rho GTPase family to nucleate actin filaments. Our results have shown that human BEG4 protein bind both G-actin and F-actin in vitro with a physiologic affinity of 0.4 mM for G-actin and 0.15 mM for F-actin. Deletion mutants of the WH2 domain fail to bind G-actin but still retain binding to filamentous actin, demonstrating the role of the WH2 domain in G-actin binding. In vitro assays have provided evidence that BEG4 promotes the creation of actin filament bundles. Moreover, overexpression of BEG4 in the mouse embryonic cell line C3H10T1/2 results in an altered actin cytoskeleton and in the production of long cytoplasmic extensions. This phenotype is maintained when we transfect a C-terminal deletion mutant, but disappears with a transfected BEG4-N terminal deleted protein. Taken together, these data show that human BEG4 is a novel protein expressed in human hair follicles and BCCs that may link the Shh signaling pathway to alterations in the actin cytoskeleton.

0775

Expression of erbB receptor family proteins in human keratinocytes and their role in UVB-induced apoptosis

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Irradiation of human keratinocytes with UVB leads to the ligand-independent activation of many cell surface receptors, including erbB1 and erbB2. However, the exact nature of this activation and the subsequent biological consequences of UVB-induced erbB receptor family activation have not been completely described. Previous studies from our laboratory have demonstrated that simultaneous inhibition of both erbB1 and erbB2 tyrosine kinase activities will suppress the induction of apoptosis resulting from UVB exposure. This protection from apoptosis was not due to the induction of keratinocytes differentiation, loss of keratinocytes viability, or inhibition of the proliferative potential of keratinocytes. To more closely examine the role of the erbB receptor family during UVB-induced apoptosis, we have generated keratinocytes cell lines which overexpress either erbB1, erbB2, or erbB3 cell surface receptors. The effect of overexpression of these UVB-targeted cell surface receptors on the response of human keratinocytes to UVB irradiation is currently under investigation.

0776

IRS1 and IRS2 have distinct roles in primary skin keratinocytes and skin fibroblasts

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The insulin receptor substrate (IRS) proteins are the first known substrates downstream to the insulin- and IGF1 receptors. Previously we have demonstrated that IRS1 and IRS2 have distinct and sometimes divergent roles in primary murine skin keratinocytes and fibroblasts. We shown that IRS1 is essential for skin differentiation while IRS2 is important for the metabolic regulation of these cells. In the present study we aimed to elucidate the molecular mechanism underlying these differences. IRS1 and IRS2 proteins are expressed and similarly tyrosine phosphorylated following insulin or IGF1 stimulation in both skin keratinocytes and fibroblasts. The next step in the signaling pathway is interaction between the IRS proteins to downstream elements including PI-3 Kinase (PI3K), Grb2 and more, leading to these proteins activation. To our surprise, in keratinocytes only IRS1 was found to interact with PI3K whereas IRS2 was not found to be associated with this protein. In contrast, in skin fibroblasts PI3K was found to associate solely with the IRS2 protein, whereas no interaction with the IRS1 protein was found. PI3K activity was associated accordingly with IRS1 in keratinocytes and with IRS2 in fibroblasts. In order to further elucidate the importance of this physical interaction we studied primary cultured skin keratinocytes and fibroblasts isolated from IRS1- and IRS2-knockout mice. We have found that the resulting consequences of the IRS1 or IRS2 inactivation were dependent on their initial interaction with the downstream elements. Whereas IRS1 inactivation was associated abnormal keratinocyte differentiation, lack of IRS2 in keratinocytes results in abnormal differentiation and a marked increased glucose uptake, whereas in fibroblasts lack of IRS2 results in a decrease in glucose uptake in both basal, insulin- and IGF1 stimulated states. Further identifying the unique roles of IRS1 and IRS2 in skin keratinocytes and fibroblasts will hopefully shed light on the complex wound healing process and the pathogenesis leading to its impairment in Diabetes.

0777

TGF-β-Smad signaling in scleroderma

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Scleroderma(SSc) is characterized by excessive synthesis and accumulation of matrix proteins in lesional tissues. Transforming growth factor-β (TGF-β) plays a central role in the pathogenesis of fibrosis by inducing and sustaining activation of fibroblasts; however, the underlying mechanisms are poorly understood. Smads are intracellular effectors of TGF-β signaling. Smad3 together with Smad4 functions as an inducible DNA-binding transcription factor that mediates TGF-β stimulation of collagen synthesis, whereas Smad7 suppresses this response. Recently we have shown that in SSc fibroblasts Smad3 and Smad4 were consistently localized within the nucleus in the absence of TGF-β in contrast to control fibroblasts and it is transcriptionaly active. In the present study we examined the levels of phosphorylated Smad2/3 and blockade of autocrine TGF-beta using dermal fibroblasts derived from SSc patients and the expression levels and description of Smads in SSc skin as well as bleomycin-induced murine model of SSc in-vivo. We found elevated phosphorylation of Smad2/3 and no effect of TGF-β neutralizing antibodies or adenovirus-mediated expression of dominant negative human Type II TGF-β receptor on Smad subcellular distribution in SSc fibroblasts, suggesting that nuclear Smad import was due to alterations downstream of the TGF-β type II receptors. Furthermore, similar with in-vitro study, both SSc patient skin and bleomycin-induced murine model of SSc showed higher staining of phospho-Smad2/3, Smad3 and Smad4 on fibroblasts and undetectable level of Smad7. To further investigate these data, transcription factor micro array are performed and it showed high Smad activity. Together, these results demonstrate constitutive activation of the intracellular TGF-β-Smad signaling as a potential mechanism contributing to the characteristic phenotype of SSc fibroblasts, and playing a role in the pathogenesis of fibrosis.

0778

Functions of EGFR during SOS-Dependent skin tumor development

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The epidermal growth factor receptor (EGFR) plays a key role in skin development and is implicated in epithelial tumor formation. Transgenic mice expressing a truncated form of the Ras activator Son of Sevenless (SOS-F) from the K5 promoter develop skin papillomas in an EGFR-dependent manner. EGFR mutant tumors are more differentiated and display an increased number of apoptotic cells, which is accompanied by decreased Akt phosphorylation indicating that the EGFR provides an important survival signal during tumor formation (1). Primary keratinocytes from K5-SOS-F transgenic mice of different EGFR backgrounds were characterized for their adhesive properties and the expression of downstream signaling molecules. We found that in K5-SOS-F transgenic keratinocytes the protein levels of Src are increased and that the adhesive and migratory capacities are altered. Moreover, the kinase domain and the C-terminal tyrosine phosphorylation sites of the EGFR are required for SOS-mediated tumor formation and efficient Akt activation. These results indicate that the EGFR is essential for oncogenic transformation by components of the Ras signaling pathway and provides a valuable target for therapeutic intervention in a broader range of tumors than previously anticipated. Therapies aimed to modulate either EGFR function or the immune system were employed in vivo on K5-SOS-F transgenic mice and the results of these experiments will be presented. (1) Sibilia et. al., Cell 102, 211-220, 2000

0779

Cultured peri-bulbar dermal sheath and dermal papilla cells induce new hair follicles from mouse epidermis and modify the growth and cycling properties of hair follicles present through natural embryogenesis

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Mesenchyme derived dermal papilla (DP) cells control development, differentiation and cycling of hair follicles. These cells are highly differentiated and non-proliferative in vivo. Significant progress has been made in defining progenitor cells of the epidermal hair follicle component but the progenitor cell source for the DP is unknown. Transgenic GFP-expressing mouse, wildtype, and non-transgenic mouse vibirssa follicle cells were cultured and implanted to CBySmn.CB17-*Prkdc*^{scid}/J mouse ears. DP-derived cells and cells from the peribulbar dermal sheath "cup" (DSC) induced new hair follicles while non-bulbar dermal sheath (DS) cells did not. Confocal microscopy revealed that GFP-expressing DSC cells induced hair growth through the formation of a new DP, DSC and the lower hair follicle DS, whereas DP cells only capable of forming a DP. In addition to inducing entirely new hair follicles, DP and DSC cells also integrated with hair follicles already present through natural embryogenesis. Biochemically, DSC cells where characterized in vivo and in vitro by low alkaline phosphatase activity in contrast to high alkaline phosphatase expression in differentiated DP cells. Thus, transplanted autogeneic and allogeneic cells derived from adult vibrissa follicles were capable of inducing new hair follicles from mouse epidermal cells and modified the growth and cycling properties of hair follicles present through natural embryogenesis. We suggest that the functional capacity of cultured DSC cells to form DP and DS and induce hair follicle development is an indicator of their pluripotency. In principle, it may be possible to utilize cultured hair follicle mesenchyme cells as a treatment for androgenetic alopecia and other forms of hair loss.

0780

Macrophage stimulating protein is a potent promoter of anagen stage hair follicles *in vitro* and *in vivo*

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Hepatocyte growth factor has been identified as a powerful promoter of hair follicle growth. In the current study we examined another HGF family member, macrophage stimulating protein (MSP) for its hair follicle modulating properties. Western blotting confirmed human dermal papilla (DP) cell expression of MSP. Immunohistology demonstrated expression of MSP receptor RON/STK in most epithelium derived follicular structures including the outer and inner root sheaths, differentiating hair matrix cells, DP, arrector pili muscle, and some cells of the sweat glands. Human hair follicles from 5 donors (2 female, 3 male, mean age 27) exposed *in vitro* for 8 days to 0.1, 1, 10, and 100 ng/ml MSP yielded a mean net increase in hair follicle length of 10.3% (n=16, P=0.0023), 12.9% (n=30, P=0.0001), 9.8% (n=18, P=0.0008), and 8.4% (n=18, P=0.0072) respectively in excess of the 43.8% mean baseline growth observed in controls (n=36). MSP was incubated with agarose beads and injected subcutaneously into mice all 70 days old when the dorsal pelage hair follicles are in a telogen resting state after completion of the second coat generation. All 8 mice receiving 1 µg MSP, and 4 of 8 receiving 100 ng MSP had hair growth induction at the site of bead implantation after 16 days while 8 mice implanted with PBS incubated beads had no hair growth response until necropsy, 22 days post injection. The data identify MSP as a potent modulator of hair follicle growth.

0781

Photo-epilation: a potential threat to wound healing in a mouse

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Many people want to remove unwanted hair for cosmetic purposes, and photo-epilations by laser or intense pulsed light (IPL) are being widely used in this field. However, to achieve permanent epilation adjacent cells to the bulge area, which are known to be a reservoir of epidermal stem cells, should be destroyed in addition to hair follicles. Thus, the possibility of aberrant wound healing after photo-epilation is strongly suspected. We wished to determine whether wound heeling capability is perturbed after photo-epilation for cosmetic hair removal. Twenty pigmented hairy C57/BL6 mice aged 5 weeks were used in this study. After wax epilation to synchronize the hair cycle, one half of the backs of mice were photo-epilated by IPL (Ellipse Flex®) in the early anagen stage. After the two hair cycles of the mice to confirm the hair removal effect, 30% trichloroacetic acid was applied to the both halves of the backs of the mice. The non photo-epilated side of the mice was shaved before the chemical peeling. A skin biopsy was performed on both sides before and just after the injury, and 2, 6, 9, and 14 days thereafter. The specimens were evaluated histologically after staining with hematoxylin and eosin, Masson trichrome, and Verhoeff-van Gieson. No differences in wound healing times were evident upon gross observation by the naked eye. However, the photo-epilated hairless skin was observed to have a thicker epidermis and dermis than normal hairy skin by histological evaluation. The cellularity of the healed wound was much denser in the photo-epilated skin and nodular condensation of fibroblasts and collagen were also observed. Collagen production of the neodermis in the normal hairy skin was first observed around the lower part of hair follicle, while it started from the upper papillary dermis in photo-epilated skin. Photo-epilation may disturb the normal wound healing process, especially dermal wound healing, and increases the risk of producing hypertropic scar or keloid.

0782

IFNγ-induced ectopic MHC class I expression in human hair matrix cells are *down*-regulated by IGF-1, TGFβ1 and αMSH: implication for hair follicle immune privilege and alopecia areata

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The anagen hair bulb displays an immune privilege (IP), since it shows low or absent MHC class I expression on the protein level. In the current study, we show by ABC, APAAP and TSA techniques as well as by *in situ* hybridization that there are substantial interindividual and location-dependent differences in the constitutive hair bulb MHC class I expression on both the protein and gene level. We have previously hypothesized that collapse of the MHC class I-dependent IP of the hair bulb is a crucial element in the pathogenesis of alopecia areata (AA) as an autoimmune disease (Paus *et al*., Yale Biol Med 1994). Since IFNγ is the key *up*-regulator of ectopic MHC class I expression in murine anagen hair bulbs in vivo (Rückert *et al*., J Invest Dermatol 1998), we have tested here whether this is also true in organ cultured human anagen scalp hair follicles. We demonstrated IFNγ (75U/ml) indeed strongly induced ectopic MHC class I expression (without triggering premature catagen development). Since effective agents remain to be identified, we then investigated the effects of several candidate *down*-regulators of IFNγ-induced MHC class I expression. Here we show by immunohistochemistry that IGF-1 (100ng/ml), TGFβ1 (30ng/ml) and αMSH (0.4μg/ml) significantly *down*-regulate IFNγ-induced ectopic MHC class I expression in human anagen hair bulb. This effect was further studied by *in situ* hybridization and RT-PCR on the mRNA level. IGF-1 also *down*-modulates MHC class I pathway molecules, e.g. TAP2 and β2-microglobulin. In addition, IGF-1 *down*-regulates not only MHC class I but also IFNγ-induced MHC class II expression. This is the first report to identify effective *down*-regulators of ectopic MHC class I expression in human hair follicles. These may prove particularly promissing therapeutic agents for the restoration of hair follicle IP in AA.

0783

Follistatin promotes the differentiation of mouse embryonic stem cells into the cells with versican promoter-driven green fluorescent protein expression

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Embryonic stem (ES) cells are pluripotent cells which can be differentiated into many cells *in vitro*. Hair follicle development is governed by the intricate signal transduction between the epidermal cells and mesenchymal cells. Hair mesenchymal dermal papilla (DP) cells expressing proteoglycan versican can induce hair growth *in vivo*. To establish an *in vitro* differentiation system of mouse ES cells into DP cells for a regenerative therapy, we have generated stably transfected mouse ES cells with a vector containing the gene of the green fluorescent protein (GFP) under control of the versican promoter. Coculture with NIH3T3 or HeLa cells induced the GFP expression in transfectants. NIH3T3 cells and HeLa cells retained this activity even after being fixed with paraformaldehyde (PFA). Furthermore, green fluorescence emitting transfectants were detected after four days serum-free culture on collagen IV coated dishes. Administration of recombinant follistatin into this culture medium significantly increased the ratio of GFP positive cells. In situ hybridization of mouse embryonic skin revealed the expression of follistatin mRNA most strongly in the hair placode and hair matrix cells. Recombinant follistatin accelerated the development of hair follicles in an embryonic mouse skin organ culture, while follistatin deficient mice showed a retardation of hair follicle formation. These results may indicate an important role of follistatin as an epidermal signal for a DP cell differentiation and hair follicle development. Our system may lead to a regenerative therapy approach for alopecia patients using their own various stem cells grown in a certain differentiating condition.

0784

Zinc pyrithione delays hair growth and follicular melanogenesis

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Zinc pyrithione (ZnPTO) is an antibacterial and antifungal agent used in some shampoos for management of dandruff. To assess the possible effect of ZnPTO on hair growth, C57Bl/6 mice were induced to a new synchronized hair cycle and subsequently treated with ZnPTO once daily throughout one hair cycle. A clear delay in hair cycle induction was observed in ZnPTO-treated mice, accompanied by a change in the appearance of the re-growing hair to a finer and more unruly hair. Histological evaluation confirmed the delay in hair growth. Striking differences in the treated mice were seen as early as day 5 of the hair cycle. At day 23, when untreated mice were already in telogen, the treated mice were still in the (delayed) growth phase. The appearance of hair pigmentation was noticeably delayed in ZnPTO-treated mice. Since follicular melanogenesis is strictly coupled to anagen, we evaluated expression levels of melanogenic proteins in treated mice. Western blot analysis showed delayed and reduced expression patterns of melanogenic genes throughout the hair cycle of treated mice. A significant decrease in tyrosinase, tyrosinase related protein-1, and dopachrome tautomerase was evident as early as day 5 of the hair cycle of treated mice. Interestingly, microphthalmia, a transcription factor that regulates tyrosinase family gene expression, was found to be expressed throughout the hair cycle in both treated and control mice. However, ZnPTO treatment significantly inhibited phosphorylation of microphthalmia, seen as early as day 3. The reduced phosphorylation of this transcription factor might be responsible for the subsequent down-regulation of tyrosinase family gene expression. Our findings suggest that ZnPTO treatment delayed the induction of a new hair cycle, resulting in shorter hair shafts at the early re-growth stage. ZnPTO may delay hair growth through its anti-proliferative activity and affects MITF phosphorylation in a proliferation-independent pathway. The precise mechanism remains to be further investigated.

0785

Hair follicles as a local reservior of skin mast cell precursors

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Hair follicles (HF) are known to serve as a "niche" for tissue regeneration. Bulge region of the outer root sheath (ORS) contains multipotent stem cells that can generate epidermis, hair follicles, and sebaceous glands, and can even give rise to melanocyte lineage. To test the hypothesis that HF may also contain precursors of skin resident leukocytes, vibrissal follicles isolated from adult mice were cultured in the presence of stem cell factor (SCF), IL-3, IL-7, GM-CSF, and Flt3 ligand. Progressive expansion of $CD45^+$ leukocytes became detectable on the top of outgrowing fibroblast layers in 10 days. When HF samples were dissected into upper fragments containing sebaceous glands, intermediate fragments containing bulge regions, and lower fragments containing hair bulb regions, leukocyte growth occurred most frequently (40-70%) from the intermediate fragments. HF-derived leukocytes showed characteristic features of mast cells: a) surface phenotype of $CD45^+$/$lineage^-$/$c\text{-}kit^+$/$Fc\varepsilon RI^+$, b) proliferative response to SCF, but not other growth factors, c) metachromatic cytoplasmic granules, d) mRNA for mast cell proteases-1, 4, 5, and 6, and e) histamine release upon FcεRI ligation or treatment with substance P or compound 48/80. They maintained these features >6 months when expanded in the presence of SCF alone. When vibrissal follicles isolated from the chimeric mice reconstituted with the GFP transgenic bone marrow cells were placed in culture, marked expansion of GFP^+ cells was observed within the HF structures and over GFP-negative fibroblasts, formally validating bone marrow origin of the HF-derived mast cells. We identified $c\text{-}kit^+$ cells within the ORS regions of vibrissal follicles and detected constitutive SCF mRNA and protein expression in the same specimens. Thus, the HF microenvironment provides two essential factors for mast cell development, i.e., progenitors expressing c-kit (SCF receptor) and local production of SCF. Our results have unveiled a previously unrecognized function of HF to serve as a local and intermediate reservoir of mast cells from bone marrow to dermis.

0786

Design and implementation of the National Alopecia Areata Registry

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A National Registry for Alopecia Areata (AA), an organ specific autoimmune disease resulting in patchy to complete hair loss, was established in Sept 2000 by NIAMS. The Registry is a web-based database for collecting epidemiology data, pedigrees, and to encode patient specimens (DNA, LB-lines, sera) from well-characterized AA subjects: multiplex families, sib-pairs, and individuals classified as persistent AU/AT or persistent patchy AA or transient AA. The registry will further genetic research by making these materials available to investigators. The Universities of Texas MD Anderson, California, Colorado, and Minnesota and Columbia University NYC are participating sites for patient examination. US residents with any form of AA, confirmed by a dermatologist, can complete a short-questionnaire about extent and duration of AA, family and autoimmunity history on line at alopeciaareataregistry.org or by mail. Patients are screened and invited to a site for extended questionnaires, exams, and blood sample collection. Dermatologists can also help in registering patients not able to travel and informing AA patients about the registry. To date, the web-site has had >44,000 hits with 800 registrants; 80% are female and 209 are children. Forty-two multiplex families and 28 sib-pairs have registered and over 70 patients have been examined and contributed samples. The existance of an Alopecia Areata Registry will serve as a resource for understanding of this complex genetic trait in the future.

0787

Fluorescence activated cell sorting of a Verapamil-sensitive, Hoechst-low side population of basal keratinocytes from the cutaneous epithelium of adult mice

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Studies directed toward the identification and isolation of keratinocyte stem cells are expected to yield important insights into the mechanisms of both epidermal homeostasis and pathogenesis of skin disease. Here, we report the identification and phenotypic characterization of a Verapamil-sensitive side population (SP) of living cells from adult murine epidermis and hair follicles following staining with Hoechst 33342. Constituting 0.02 to 0.09 percent of viable cells, the SP from cutaneous epithelial cells is similar to that of hematopoietic stem cells and several other tissues. Phenotypic characterization of epithelial SP cells demonstrated that virtually all of them expressed both keratin 14 and alpha-6 integrin proteins, indicating that they are basal keratinocytes. However, immunocytochemistry of SP cells detected only low expression of keratin-6 (approximately 30%), a marker of hair follicles. Although CD34 protein was not detected, CD34 message was present by RT-PCR. We have undertaken studies to determine the stem/progenitor characteristics of epidermal SP cells: whether they are quiescent and retain [3H] thymidine label (slowly cycling label-retaining cells), are clonogenic in vitro, or reconstitute a graft. In summary, our initial characterization of SP cells from the cutaneous epithelium of adult mice indicated that they are basal keratinocytes; however, the relative paucity of keratin 6 or CD34 expression suggests that some SP cells may not originate from the bulge region of the hair follicles. Thus, the SP may include both bulge and non-bulge progenitors, a concept supported by cell kinetic studies, mathematical models, and carcinogenesis experiments suggesting potent progenitors exist in the epidermis as well as the hair follicles.

0788

Regulation of human hair follicle vascular responses and growth by neurokinin 1 receptor ligands

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Angiogenesis is an important process in hair follicle growth. The formation of new vessels and the remodelling of existing vessels are likely to be induced by the major endothelial cell-specific stimulatory factor called VEGF. Recent reports revealed that neural mechanisms play a role in hair growth control. It has been also demonstrated the presence of a close spatial localization of vascular network and substance P in hair follicle. To understand the nature of the neural and vascular interactions in human hair follicle we investigate the relationship between the neurokinin-1 (NK1) receptor ligands and VEGF receptors expression in human hair follicle and the impact of these interactions on the regulation of hair follicle growth. We demonstrated the presence of NK1 receptors in human hair follicle cells. The anti hair loss drugs, minoxidil and finasteride, enhanced NK1 receptors protein and gene expression in human hair follicle. We also observed that substance P up-regulates VEGF receptors in human hair dermal papilla cells (HDPC) and in isolated human hair follicles in anagen phase. By the use of selective NK1 receptors agonist [Sar9,Met (O2)11] substance P, it was established that the activation of NK1 receptors is most likely to mediate the angiogenic responses induced by substance P. The angiogenic activity of SP was abolished by treatment with the selective NK1 receptors antagonist (L-732,138). Ex vivo studies indicate that NK1 receptor antagonist also inhibited the rate of hair follicle growth. These results demonstrate the interactions existing between angiogenic factors and neurokinin 1 receptor activity in hair follicle. The increase of hair growth rate in the presence of NK1 receptor agonist is associated with a stimulation of VEGF receptors. These data may be a key to new advances in the field of hair loss treatments with a particular emphasis on the alopecia areata disease associated with a decrease of substance P expression.

0789

Direct evidence that head and rod domain sequences interact to control states of assembly of trichocyte keratin intermediate filaments

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Keratin intermediate filaments (KIF) are assembled in a series of hierarchical steps from: a single protein chain; a dimer coiled-coil molecule; a tetramer consisting of a pair of antiparallel partially staggered molecules; a unit length filament (ulf) entity consisting of about 8 tetramers aligned side-by-side and about 20 nm wide; as the ulfs join end-to-end, the assembly begins to shrink to 10 nm as the molecular strands entwine; and finally a uniformly-wide 10 nm KIF is formed. However, many details of this model have yet to be proven experimentally. In particular, we want to know what is the precise role of the head domains in these states of assembly. We replaced each arginine residue of the type I and II trichocyte chains one at a time with a lysine, and used full-length as well as truncation and deletion forms to explore molecular assemblies by cross-linking with DST (0.6 nm arm) or BSOCOES (1.3 nm). We found that the head domain of the type I chain interacts with the 1A, L1 and early 1B segments of the rod domain. The head domain of the type II chain engaged in similar interactions, but primarily with the late 1B, 2A-L2-early 2B rod domain segments. Thus the head domain of the type II chain is especially important as it can adopt multiple interactions along the rod domains of molecules in adjacent molecular strands. In stable ulf particles formed in the absence of the beta-strand motif on the head domain of the type II chain, there were no interactions of the type II head and 2B rod domain segments. This may be because the molecular alignments in such ulfs are not the same as those in intact KIF. Together with previous work in which we have mapped possible functional domains on the head domains, these new data document that direct interactions between the head and rod domain are responsible for various levels of structural hierarchy within KIF. This will now allow modeling on how molecular strands pack within KIF.

0790

Identification of essential motifs on head domain sequences involved in the assembly of trichocyte keratin intermediate filaments

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The head domain of intermediate filament (IF) protein chains is essential for assembly in vitro and in vivo. For example, headless type I or II keratin or type III vimentin chains do not assemble. While portions and structural motifs in the vimentin head domain have been described, there are few comparable data for keratin IF. We have made constructs with the type I and II mouse trichocyte keratin chains. Selective truncations or deletions were made, and then used to map essential regions for each hierarchical stage of IF assembly. The first 9 residues in either chain are not essential for assembly. Residues 10-21 in both chains possess a conserved motif that is essential for unit-length filament (ulf) stability; its absence from either chain arrests KIF assembly at the tetramer-octamer stage. Residues 22-49 (type I) or 22-31 (type II) consisting of irregular linker sequences, are similarly required for ulf stability. Residues 32-67 (type II) contain four potential beta-turn motifs that are essential for ulf elongation, as their absence fosters formation of stable ulf. Cross-linking studies show these ulf consist of alternating rows of molecules in the A11 and A22 alignment modes, interfaced by the A12 mode, to produce the characteristic 20-nm wide ulf raft, but the A22 and A12 alignments are set further apart than in intact KIF. Thus the beta-turn motifs on adjacent type II chains promote normal A11, A12, A22, and ACN interactions for KIF elongation. Residues 49-57 (type I) and 70-104 (type II), containing the respective H1 regions, are required for tetramer-octamer stability, as their absence in whole or in part allows only homo- or hetero-tetramer formation. These data offer the first systematic analysis of the roles of various portions of head domain sequences of a keratin chain system. Similar motifs may exist on other keratins, but are not easily recognizable due to the insoluble and refractory nature of the glycine-rich sequences.

0791

Rules of hair cycling and skin domains revealed by Msx2 knockout mice

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We recently reported the skin phenotype of Msx2 homeobox gene knock out mice (Development 130, 379-389). Analyses demonstrated that the hair follicles of these mice have shorter anagen, longer catagen, prolonged telogen and precocious exogen. As a result, the mice show a characteristic *cyclic alopecia* phenotype with complex and dynamic alopecic and hairy skin patches. Previously researchers analyzed hair cycling in single hair follicles, we now analyze the social behavior of hair cycling in groups of hairs. The following rules are observed. 1) Hairs are usually distributed into domains: cranial, cervical and several trunk domains. Each domain has its own hair cycle pace, asynchronous with other domains. Domains split gradually with increasing age. In adults, the domains further split into one central and two lateral domains. The position and shape of lateral domains are usually bilaterally symmetric. These skin domains are not the result of Msx2 loss of function, as they also exist in normal mice as revealed by shaving and tracking the reappearance pattern of anagen hairs. 2) For hair cycling of each domain, there is an *initiation center* which starts anagen, *spreading wave* which occurs in all directions, and *borders* that are refractive to spreading. 3) The domains are not stable. From cycle to cycle, the location of the initiation centers and the border of domains can change even on the same mouse, suggesting stochastic events are in place. Sometimes, one domain can have multiple initiation centers. Sometimes, two domains can fuse into one. As a result, domain maps of different mice are similar but not identical. A time-lapse cartoon movie is prepared to show the dynamics. 4) For hairs of the same region, anagen duration is more constant and telogen duration is more variable. Traditional telogen can be divided into an early phase when skin is refractive to anagen initiation, and a later phase when skin is responsive to anagen initiation signals. The molecular bases of these hair follicle behaviors are undergoing current investigation.

0792

Tiger tail banding and hair shaft abnormalities in trichothiodystrophy and other hair disorders

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Trichothiodystrophy (TTD) is a rare autosomal recessive disorder characterized by short, brittle hair with low cystine content and alternating dark and light (tiger tail) bands under polarizing microscopy. Some TTD patients also show ichthyosis, intellectual impairment, decreased fertility, and short stature (IBIDS). Others have, in addition, photosensitivity (PIBIDS). TTD patients do not have a predisposition to skin cancer. The findings in xeroderma pigmentosum (XP) are markedly different, with acute sun sensitivity, lentiginous pigmentation, poikiloderma, and a 1000-fold increase of UV induced skin cancers. Most PIBIDS patients have a mutation in either of two XP DNA repair genes: *XPD* or *XPB*. Rarely, TTD may also occur in combination with XP. We examined hairs from 10 TTD patients and 4 XP-TTD patients for tiger tail banding and hair shaft abnormalities. Using a rotating microscope stage, virtually all hairs in 14 samples showed tiger tail banding under polarizing light. All 14 had hair shaft abnormalities but the proportion of involved hairs varied among the patients. These included surface irregularities (13/14), trichoschisis (12/14), and trichorrhexis nodosa-like defects (7/14). Based on amino acid analysis, the 4 patients with XP-TTD had cystine content intermediate between normal and TTD levels. These patients had fewer hair shaft abnormalities than 2 TTD siblings with low cystine content. In contrast, an XP-D and an XP-A patient did not show tiger tail banding or hair shaft abnormalities and had normal sulfur amino acid analysis. We also examined control hairs from 7 normals and 50 patients with XP-C or other dermatologic conditions, including a variety of disorders of cornification. None of these hairs showed tiger tail banding or the described TTD hair shaft abnormalities. Tiger tail banding on polarizing microscopy and the presence of certain hair shaft abnormalities provide a reliable method for rapid bedside diagnosis of TTD.

0793

Stimulation of hair growth in a C3H/HeN mouse model by topical application of ketoconazole

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Ketoconazole (KCZ) is an imidazole anti-mycotic agent, and its topical application is also effective for treating seborrheic dermatitis and dandruff. Recently, topical use of KCZ 2% shampoo was reported clinically to have a therapeutic effect for androgenic alopecia. The present study was conducted to examine statistically the stimulatory effect of KCZ on hair growth in a mouse model. Coat hairs on the dorsal skin of seven week old male C3H/HeN mice were gently clipped, and either 2% KCZ solution in ethanol (EtOH) or 1% EtOH was topically applied once daily for three weeks. The clipped area was photographed every other day, and the ratio of re-grown coat area was then calculated. The results demonstrated that KCZ had a clinically significant stimulatory effect on days 15, 18 and 21 compared with the vehicle group ($p<0.05$, n=8). Repeated experiments showed similar effects, confirming the efficacy of KCZ as a hair growth stimulant. Although the therapeutic mechanism of KCZ for hair growth is unclear at this moment, our results suggest that topical applications of the substance are useful for treating seborrheic dermatitis accompanied by hair regression or androgenetic alopecia.

0794

Gap junction formation in human fetal hair follicle and its bulge region

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Gap junctions (GJs), composed of connexins (Cxs), are channels allowing intercellular communication, and play a key role in the regulation of cell proliferation and differentiation. The Cx expression pattern and GJ formation in human fetal hair follicle (HF), including bulge that is prominent in fetal HF and is believed to be stem cell-rich site, have not yet been clarified. We used immunofluorescent labeling to investigate the sequential expression pattern of Cx26 and Cx43 in developing HFs in human fetuses of a series of estimated gestational age (EGA) (49-163 days). Ultrastructural GJ formation was observed by electron microscopy and the numbers of GJs were quantitatively analyzed. Both Cx26 and Cx43 expression were observed at 88 days EGA in the hair peg. At 135 days EGA, Cx26 was expressed in the outer root sheath (ORS) and the inner root sheath (IRS), while Cx43 expression was chiefly in the IRS, hair matrix and sebaceous glands. At 163 days EGA, Cx26 expression in the ORS was most intense in the outermost layer, in contrast to Cx43 expression in the inner part of ORS. In the bulge, only Cx43 was expressed in a few cells. Ultrastructurally, GJs were observed at 102 days EGA in both the ORS and IRS. The numbers of GJs per cell were 0.03 at 102 days EGA, 0.05 at 120 days EGA and 0.11 at 152 days EGA, increasing over time. GJs were also observed in the bulge, and the numbers per cell were 0.04 at 120 days EGA and 0.08 at 152 days EGA, which were only about 20% to 30% less than those of ORS and IRS cells. The changing expression patterns of Cx26 and Cx43 and increasing GJ formation suggest the close association of Cx expression and GJ formation with HF morphogenesis. In addition, the ultrastructural observations showed that the considerable number of the bulge cells, the putative stem cells, form GJs during human HF development.

0795

Bone morphogenetic protein receptor IA is expressed in developing human sebaceous gland

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BMPs comprise a family of secreted signaling molecules which have multiple functions during animal development, including mouse hair follicle morphogenesis. However, little is known about the regulation of human fetal hair follicle morphogenesis by BMP signaling. We studied the distribution patterns of BMP 2 & 4, BMP receptor IA (BMPRIA) and several signal molecules by confocal laser scanning microscopy in human embryonic and fetal skin specimens at 49-163 days estimated gestational age (EGA). Strong BMPRIA labelings were seen in the human fetal sebaceous gland (SG) primordium at 108 days EGA and after. At 135 days and after, cells of the developing arrector pili muscle as well as SG primordium were positive for BMPRIA. In adult human hair follicle, the SG turned to be negative for BMPRIA, although the arrector pili muscle expressed BMPRIA strongly. High power view revealed that the BMPRIA was distributed mainly on the cell periphery and in the perinuclear endoplasmic reticulum areas of the human fetal SG primordium cells. Smad1/5/8 was expressed strongly in the mesenchymal cells around the SG primordium. In addition, in the SG primordium, immunolabeling of Smad1/5/8 was observed mainly in the nuclei. No apparent immunoreactivitiy for BMPRIA was seen in other portion of developing human hair follicle, including the bulb region. No significant expression was detected for BMP2 or BMP4 throughout hair follicle development. Lef-1 was expressed in the hair peg and in the inner root sheath of developing human fetal hair follicles. The BMPRIA expression may suggest the binding of exogenous BMPs to the SG primordium cells, although it was not possible to detect BMPs in and around the developing SG in the present study, most likely due to low levels of these proteins in the extracellular space. Furthermore, smad1/5/8, the molecule in the downstream of BMP signaling, was expressed in the SG primordium cells. Our observations suggest the involvement of BMP signaling via BMPRIA in the antiproliferative/differentiation programmes of the SG primordium cells.

0796

Towards the development of safe, topical cyclosporine derivatives as hair-growth-stimulators

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Since hypertrichosis is one of the leading undesired effects of systemic cyclosporine A (CsA) therapy in man and since topical and systemic CsA potently induces anagen and inhibits catagen in mice, CsA is the most attractive candidate "hair drug" for human alopecias. However, the serious side effects associated with systemic CsA administration far outweigh the potential trichological benefits. Therefore, topically applicable and, ideally, non-toxic CsA derivatives deserve to be developed as a hair growth stimulators for clinical use. In the current studies, we have systematically explored whether it is possible to modify the CsA molecule in such a way as to maintain its hair growth-modulatory potency while significantly reducing its unwanted nephrotoxicity, which is the major dose-limiting adverse effect of long-term CsA administration, and immunosuppressive effects. Initially we have screened 6 candidates, LG 40, 64-1, 70-2, 409, 432, and 474, based on the their anagen-inducing potency in the C57BL/6 mouse model. The LG 70-2 was an equipotent anagen inducer compared to CsA and retarded cyclophosphamide-induced massive, premature catagen development. In organ-cultured human occipital scalp hair follicles, LG 70-2 or CsA stimulated hair shaft production, as measured by ^{35}S-cysteine incorporation. Oral administration of CsA caused significant renal toxicity, evidenced by the increases in the serum creatine and urea concentration, and a decrease in the magnesium concentration, while there were no significant changes in these parameters in rats treated with LG 70-2 and other analogs. When CsA was administrated to 40 mice at a dose of 3g/kg all animals were killed, while none of the mice treated with LG 70-2 and other derivatives was killed. The MLR (mixed allogenic lymphocyte reaction) data for LG analogs widely ranged from 30 % to less than 2 % of the IC 50 of CsA. This encourages to further explore less toxic LG analogs as the most interesting candidates for hair drugs for topical application.

0797

Identification and expression analysis of a second domain of human high sulfur keratin-associated protein genes on chromosome 21q23

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Analysis of the EBI/ Genebank database using nonhuman keratin associated protein (KAP) gene sequences as a query resulted in the identification of two domains of KAP genes on chromosome 21, one of which was recently characterized (Rogers et al. JBC 227: 48993-49002, 2002). The second domain, a ca. 90 kb region on human chromosome 21q23 harbours sixteen KAP genes and two KAP pseudogenes. These genes could be divided into two families, twelve members of which are the human orthologs of the sheep KAP 10 family, and four members which are orthologs of the mouse KAP12 family. Systematic cDNA/ 3-RACE isolation studies, as well as in situ hybridisation analysis of all of the KAP genes identified showed the mRNA expression of a majority of the members of both families in a narrow region of the hair fiber cuticle.

0798

***EDA* function in skin appendage formation revealed by conditional transgenic mice**

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Mutations in the X-linked *EDA* gene cause Anhidrotic Ectodermal Dysplasia (EDA) with lesions in skin appendage formation; mutations in mouse EDA give similar "Tabby" phenotype. Among several EDA splice isoforms, the longest, EDA-A1 and EDA-A2, which belong to the TNF ligand family, activate NF-kB transcription factors through corresponding receptors, EDAR and XEDAR. To get clues to the function of EDA in skin appendage formation, we generated Tet-regulated conditionally expressed EDA-A1 and EDA-A2 isoforms as transgene in Tabby and wild-type mice. Here we show that the expression of the EDA-A1 transgene fully restored guard hair follicles and sweat glands, but did not restore zigzag hair, and instead strongly suppressed zigzag hair follicle formation in wild type mice. In addition, marked hyperplasia of sebaceous glands was observed in response to high levels of EDA-A1 in transgenic Tabby mice, and was reversed when transgene expression was turned off. In contrast to the effects of EDA-A1, the EDA-A2 transgene restored neither hair follicles nor sweat glands, and had no effect on sebaceous glands, indicating that it likely functions in cooperation with other isoforms. The results reveal isoform-specific actions during skin appendage formation, and further suggest possible interactions of different EDA isoforms.

0799

The *in vitro* differences between hair follicle bulge stem cells and hair bulb keratinocytes

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The goals of this study are to define the in vitro characteristics of keratinocyte stem cells, isolated from the bulge region of human hair follicles, and determine how they differ from the transit-amplifying (TA) cells from the hair bulb. We used telogen follicles, plucked from dispase-treated human scalp, to generate primary keratinocyte cultures from this stem cell-enriched area. We also dissected the bulb region of anagen follicles to obtain matrix/TA keratinocytes. We then compared the in vitro properties of cell adhesion, cell migration, clonogenicity and in vitro life-span for these two populations. We observed epithelial outgrowths from the bulbs within two days of explant, consistent with the active proliferation of TA cells, while telogen outgrowths appeared between 7-10 days after explant, reflecting their normal in vivo quiescent nature. After growing for two weeks, the cells were re-plated and analyzed. Both populations formed colonies, however the stem cells from telogen follicles formed more colonies than bulb keratinocytes (147+/- 14 vs 83 +/- 9), and more colonies > 3 mm (41% vs 23%). When the cells were sub-cultured, telogen cells formed colonies until passage 6 when they only formed terminally differentiated cells, while bulb cells terminally differentiated at passage 3. On cell adhesion assays, the ratio of cells rapidly adhering to collagen IV within 10 min. was greater in stem cells vs. TA cells, 84.6% vs 55% respectively. Most strikingly, bulb keratinocytes showed a 7-fold greater mobility on migration assays than the telogen bulge stem cells (.704 vs .102 microns/min.) Our results show distinct in vitro differences between stem cells from the telogen bulge and TA cells from the anagen bulb. These assays thus help to define the hair follicle stem cell phenotype and correlate with the in vivo properties of stem cells such as permanent residence within the stem cell niche, tightly adherent to the basement membrane. The assays can also now be used to determine the molecular requirements which maintain the differences between stem and TA cells.

0800

Inhibition of PKC activity slows hair growth: possible involvement of COX2

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The expression of cyclooxygenase 2 (COX2) mRNA was shown to be increased in depilation-induced anagen phase of the hair cycle, mainly in the outer root sheath cells. Protein kinase C (PKC) appears to regulate COX2 expression in that TPA, known to first activate and later deplete PKC, induces the protein level of COX2 in both mouse and human keratinocytes. We have observed that the topical application of a selective PKC inhibitor Bisindolylmaleimide (300μM) (Bis) caused shortening and thinning of hair in mouse and guinea pigs after 5 weeks twice daily application. In order to explore the role of COX2 in mediating this effect of PKC inhibition, sections from biopsies taken from areas of hair regrowth on the backs of guinea pigs at end of the experiment were stained using an antibody against COX2. COX2 staining was strongest in the outer root sheath cells, as previously reported, but less in Bis-treated areas than in vehicle-treated areas. To further explore whether Bis-induced changes in hair growth may involve COX2, paired cultures of primary human keratinocytes were treated with 10^{-7}M TPA or vehicle alone, or 100μM Bis alone or Bis plus TPA for 24 or 48 hours. Within 24 hours of treatment, TPA induced a dramatic differentiation of keratinocytes as judged by culture morphology, as previously reported. However, the PKC inhibitor Bis completely blocked TPA-induced differentiation. Immunoblot analysis using cells harvested after 48 hours showed that TPA induced COX2 protein, as previously reported, and this induction was completely blocked by simultaneous treatment with Bis. These results suggest that initial PKC activation, rather than its subsequent depletion by TPA, is responsible for COX2 inhibition. Further, the data suggest a role for COX2 in regulating hair growth.

0801

WNT signals in the initiation of epidermal appendage development

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Stem cells in the embryonic epidermis give rise to several types of appendages, including hair, mammary glands and teeth, in addition to the epidermis. We have previously shown that WNT paracrine intercellular signaling molecules are required for the initiation of hair follicle development. We hypothesize that WNTs are generally required for embryonic epidermal stem cells to adopt an appendageal fate. To test this hypothesis we examined WNT pathway gene expression during development of the mammary streak and mammary buds. Of multiple Wnt genes surveyed, we found that Wnt10b shows the earliest specific localization to the mammary region, being expressed in the mammary streak epithelium and in the mammary buds as they develop. Wnt6 is strongly expressed in mammary buds at slightly later stages, and Wnt4, Wnt5a, Wnt7a, Wnt10a, and Wnt11 are expressed at lower levels. We find that WNT pathway genes such as beta-catenin, Kremen1, and Kremen2, and Lmx1, a known target of Wnt7a, are expressed at early stages of bud development. To determine whether inhibition of WNT signaling blocks the initiation of mammary streak or bud development we examined the mammary region in transgenic embryos expressing high levels of the potent WNT inhibitor Dickkopf 1 (DKK1) in the surface epithelium. These embryos lack any histological sign of mammary bud formation. To determine the precise stage at which mammary bud development is arrested we examined expression of Wnt10b, beta-catenin and Fgf10, an early regulator of mammary streak formation, in DKK1-expressing and control embryos. We find that DKK1 prevents localized expression of Wnt10b and beta-catenin in the mammary region epithelium. However, expression of Fgf10 in the mesenchyme underlying the presumptive mammary streak is unaffected by Dkk1 at E10. These results suggest that WNT signals are required downstream of Fgf10 for the initiation of mammary streak development, and support the hypothesis that WNTs are necessary for epidermal stem cells to adopt an appendageal fate.

0802

Identification and characterization of cornifelin, a novel cornified envelope component, related to psoriasis

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To identify psoriasis-related genes, we compared mRNA populations of normal and psoriatic skin by random 3'-directed tag sequencing from non-biased cDNA libraries. We selected one of candidate genes, designated as cornifelin, which showed the increased expression in psoriatic skin. Cornifelin contains 112 amino acids with a predicted molecular weight of 12367 Da and is expressed only in uterus, cervix, and fetal skin by Northern blot. Cornifelin transcript was expressed in the granular cell layers of the epidermis in uninvolved psoriatic skin, and widely detected in the upper-spinous layers in psoriatic skin, but not in normal skin, by in situ hybridization analysis using a cornifelin riboprobe. To investigate the function of cornifelin, we established a transgenic mouse line overexpressing human cornifelin in the epidermis using a human involucrin promoter. Cornifelin protein was detected in both protein fractions precipitated with anti-loricrin and anti-involucrin antibodies using the specific antibody against cornifelin, suggesting that cornifelin is directly or indirectly cross-linked to other cornified envelope proteins, loricrin and involucrin. Transgenic mice of cornifelin showed reduced expression of loricrin and enhanced expression of involucrin without altered expressions of K6, K10, and K14 and other morphological changes compared to wild type mice. In this study, we found a novel cornified envelope component, cornifelin, which may be involved in the pathological processes of psoriatic epidermis.

0803

Caspase-9 pathway is involved in the induction of apoptosis by TGF-beta: a possible catagen mechanism in human hair cycle

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We have already shown that TGF-beta2 plays an essential role in the induction of the regressing phase, catagen, during the human hair cycle. In this study, we analyzed mechanism of apoptotic cell death induced by TGF-beta2. mRNA processed to cDNA from immortalized outer root sheath cells (IORS) were compared with or without 25 ng/ml of TGF-beta2 at 30 h using Clontech human plastic 83K array. TGF-beta2 induced variety of signal transduction molecules including kinases and phosphatases. Proteasome componens were up-regulated, whereas matrix metallo proteases were down-regulated. Interestingly upregulation of caspase-9 and down regulation of caspase-8 were noticed. For the confirmation of microarray results, real-time PCR was carried our using LightCycler system. Caspase-9 and caspase-3 mRNAs were gradually increased with time reaching 3-fold increase at 48 h in the presence of TGF-beta2. Expression of caspase-8 was slightly decreased or not altered significantly. Essentially the same results were obtained for the primary ORS cells. Immunohistochemical study demonstrated that active caspase-9 and caspase-3 were localized in the regressing ORS cells in the transient stages of human catagen hair follicles in vivo. Furthermore hair follicles cultured with TGF-beta2 showed activation of these caspases in ORS cells. Collectively our results indicate the catagen induction mechanism as follows. TGF-beta2, that is upregulated during the transition phase, triggers synthesis and accumulation of caspas-9 and 3, resulting the activation of intrinsic caspase network to eliminate follicular keratinocytes.

0804

Impaired sonic hedghog signaling and defective hair germ formation and elongation in laminin 10 deficient mouse

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Mesenchymal-epithelial interaction plays a critical role during hair follicle development. The defect of hair germ formation and elongation in laminin 10 (alpha5beta1gamma1) deficient (lam a5-/-) mouse suggests an important role of laminin 10 in this process. Sonic hedgehog (Shh) signaling has been implicated in hair germ invagination and elongation downwards into the dermis. In this study, we examined the mRNA expressions of Shh and its downstream effector Gli 1 in laminin 10 deficient mouse skin hair follicles using in situ hybridazation with [^{35}S]-UTP-labeled Shh and Gli 1 specific riboprobes. Due to the fact that laminin 10 homozygous knockout (lam a5-/-) mouse embryos die at about gestation day 16.5 (E16.5), we studied skins from the embryo E16.5 and their grafts that had been grafted onto nude mice for 5 days. While Shh and Gli 1 expressions were observed in wild type skin localized to hair germs in E16.5 skin and elongating hair germs in 5-day grafts, a dramatic decreased expression of Shh or Gli 1 was noted in mutant skin of the embryo or graft samples. Semi-quantitative and statistical analyses confirmed significant difference in the mRNA expression levels of Shh and Gli 1 in the wild type and mutant skins. Our study indicates that the defect in the formation of hair germ and failure of hair germ elongation in laminin 10 deficient skin are likely the direct result of impaired Shh signaling. The data also suggests that normal expression of laminin 10 may be required in part in the activation of Shh signaling pathway during hair follicle development.

0805

The zebrafish: a vertebrate genetic model for studying cutaneous biology

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The zebrafish has emerged as an important vertebrate genetic model for the study of numerous developmental and biological processes. Our recent investigations have focused on neural crest specification and scale development utilizing zebrafish mutants. Melanocytes are a derivative of the neural crest. Three zebrafish mutants exhibit interesting abnormal neural crest phenotypes: swirl (swr/bmp2b) with greatly reduced or absent neural crest progenitors (NCP); somitabun (sbn/smad5) with a large expansion in the number of NCP; and snailhouse (snh/bmp7) with a moderate expansion. All three are members of the Bone Morphogenetic Protein (BMP) signaling pathway. We show evidence that different residual levels of BMP signaling exist in these mutants. We propose that low levels of BMP signaling, as is likely found throughout most of the sbn/smad5 mutant embryo, specifies NCP from presumptive naive ectoderm. We put forth a model to explain the NCP phenotypes displayed by these mutants in terms of levels of BMP signaling. We tested this model by several approaches: overexpression of mRNA encoding various members and inhibitor of the BMP pathway in order to change the level of BMP signaling in mutants/wild-type embryos; antisense/Morpholino technology; and Western Blot analysis using an antibody specific for the activated form of Smad 1/5, downstream members of the BMP pathway. Our results are consistent with our proposed model. We also have begun analyzing the development of scales in the zebrafish. Our gene expression analysis have shown evidence for various signaling pathways, including Wnt, Sonic Hedgehog, BMP, and Notch/Delta in the development of scale placodes, as reported for hair. Analysis of a scaleless mutant also has begun.

0806

Epithelial and mesenchymal mitogens stimulate VEGF expression in cultured dermal papilla cells

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Vascular Endothelial Growth Factor (VEGF) acting selectively on endothelial cells induces angiogenesis and enhances vascular permeability. In previous studies we found, that human cultured dermal papilla cells synthesize VEGF, implicating its role in hair vascularisation. Moreover VEGF synthesis was influenced *in vitro* by addition of proinflammatory cytokines suggesting possible link between vascular supply and the development of inflammatory processes involving human hair follicle. The aim of this study was to find the relationship between hair cycle regulating growth factors and VEGF synthesis from hair papilla. Several growth factors: EGF, TGFα, TGFβ-1, bFGF, PDGFaa, IGF-1 and NGFβ, mitogens for various epithelial and mesenchymal cells and influencing hair growth cycle were investigated for their effect on VEGF gene expression and protein synthesis in cultured dermal papilla cells. VEGF mRNA expression was measured by semiquantitative RT-PCR method and VEGF proteins by radioimmunoprecipitation. Growth factors known to be promoters of angiogenesis like TGFβ1, PDGFaa as well as anagen-promoting IGF-I significantly stimulated VEGF mRNA expression in dermal papilla cells. The highest VEGF mRNA levels in DPC were observed after stimulation with EGF and IGF-1 after 2 hours. Hair cycle-dependent vascular changes are probably regulated by the same cytokines and growth factors, which control hair growth cycle, and are synchronized with the growth of other follicular structures. The investigation of VEGF expression in DPC *in vitro* may serve as a model of influencing angiogenetic processes associated with human hair follicle, and therefore might be potentially useful in finding new treatment options of alopecia.

0807

Development of gene-switch-Smad7 transgenic mice for studying the molecular mechanisms of Smad7 in skin development

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Smad7 is a signaling inhibitor of the TGFβ super family. Previously, we have generated Smad7 transgenic mice, using a keratin 5 (K5) promoter. K5.Smad7 mice exhibit a severe delay in hair follicle development and die at neonatal stage. Consistently, K5.Smad7 transgenic skin exhibited reduced β-catenin protein. However, it is not clear whether this change is directly induced by Smad7 or the consequence of pathological changes. Additionally, the early death of K5.Smad7 founders precludes them to be used in studying the functions of Smad7 during hair follicle development in embryos and hair cycling in adults. Therefore, we have generated gene-switch-Smad7 mice in which Smad7 transgene expression can be induced by application of RU486. At day 14 pregnancy, mice carrying gene-switch-Smad7 embryos were injected s.c. with RU486. The embryos were dissected at E18.5. Hair follicles in Smad7 transgenic embryos were at the stage equivalent to E14.5 in normal embryos, indicating that Smad7 overexpression blocks hair follicle development at the earliest stage. Twelve hours after single topical application of RU486 to gene-switch-Smad7 mouse skin resulted in a significant reduction of β-catenin protein. Thus, down regulation of β-catenin is likely a direct effect of Smad7 overexpression and contributes to delayed and aberrant hair follicle development. To study the role of Smad7 in hair cycling, Smad7 overexpression was induced at different stages of hair follicle cycling. After Smad7 transgene induction, hair follicles exhibited a significant delay in the transition from telogen to anagen. In addition, apoptosis at the categen phase was blocked after acute Smad7 induction, suggesting that blocking TGFβ1-induced apoptosis is one of the mechanisms by which Smad7 delays hair follicle cycling. Our results show that the gene-switch-Smad7 transgenic mice are a unique model to delineate the functions and molecular mechanisms of Smad7 in the skin.

0808

A novel WNT agonist is required for epithelial-mesenchymal interactions in the hair follicle

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There is an absolute requirement for reciprocal signalling in the maintenance of epithelial-mesenchymal interactions during hair follicle morphogenesis and cycling. Secreted factors of the WNT/Wingless family have been implicated in both the first epithelial message and also in maintenance of inductive ability in the mesenchyme. In the adult hair follicle, anagen is maintained by the interaction between the mesenchymal condensation known as the dermal papilla (DP) and the overlying proliferative matrix cells, while the dermal sheath (DS) is physically isolated from signals from the hair follicle epithelium. We performed gene expression analysis between rat vibrissae DP and DS with the hypothesis that genes expressed in the DP but not in the DS may be specifically determined by the interaction with the overlying epithelial cells. A number of genes are induced in the DP as a result of the interaction, the most prominently induced gene being the uterine sensitivity associated protein, USAG-1. During anagen, this secreted factor is expressed in a domain confined to the region of epithelial-mesenchymal interaction in the hair follicle and by RT-PCR we show that the expression of USAG-1 falls rapidly when the DP cells are cultured. In vivo experiments in Xenopus laevis embryos and in vitro transfection experiments on cultured NIH3T3 cells show that USAG-1 can enhance signalling by specific WNTs while concomitantly down regulating signalling by the TGFb superfamily of genes. The same WNTs are expressed in the hair follicle during anagen, as are their co-expressed secreted inhibitors, the secreted frizzled-related proteins (sFRPs). We hypothesize that USAG-1 modulates the interaction between the WNTs and sFRPs to control not only hair follicle differentiation, but epithelial-mesenchymal interactions in general.

0809

Prolactin regulates onset of anagen and size of underhair follicles in mink (*Mustela vison*)

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In mink, ♠ 10-20 hair follicles develop from a common follicular canal into bundles of; *G-type*: one large guard hair plus a variable number of underhairs, *I-type*: one intermediate size guard hair plus a variable number of underhairs, and *U-type*: underhairs only. Winter anagen is correlated with reduced serum prolactin (PRL) levels, and maximum hair density, whereas summer anagen is correlated with elevated serum PRL levels and minimum hair density. Our objectives were to determine if PRL has effects on anagen initiation, hair diameter and/or density, and if the effects are mediated equally between fiber or bundle type (G, I or U). Mink (4/group) in telogen (Feb 20), were treated as; Haloperidol (HAL), Melatonin (MEL), or Controls, and the dorsal skin plucked of hair to induce anagen. Onset of anagen in HAL (Mar 27) and MEL (Mar 29) treated mink occurred earlier than controls (April 9; P•0.05). Serum PRL levels were 5.6 ± 0.91 ng/ml (Control), 7.8 ± 1.1 ng/ml (HAL; P•0.05), and undetectable in MEL-treated mink. Density of I-Guard hairs (4.93 ± 0.05 / mm^2) was greater than G-guard hairs (3.15 ± 0.05 / mm^2; P•0.05), but the diameter of G-guard hairs (127.5 ± 1.53 μm) was greater than I-type (51.0 ± 0.34 μm; P•0.01). There was no difference in guard hair density or diameter in response to treatments. Density of underhairs in all bundle types was reduced by HAL and increased by MEL (P•0.05). Underhair diameters did not differ between G, I & U bundles within treatments groups, but were increased in response to HAL (18.7 ± 0.78 μm) and decreased by MEL (9.5 ± 0.56 μm) compared to controls (14.1 ± 0.082 μm; P•0.01). Finally, underhair diameters of mink in spontaneous summer anagen (13.0 ± 0.14 μm) were greater than those of mink in winter anagen (10.6 ± 0.14 μm; P•0.001). We propose that during the summer, PRL initiates anagen and increases follicular size in a portion of the underhair follicles, which results in reduced fur density. In contrast, during winter, low PRL levels results in reduced follicular size, but a greater number of follicles entering anagen.

0810

TAp63α induces a squamous epithelial program but blocks terminal differentiation

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Expression of p63, a transcription factor that is transcribed into six isoforms, is required for proper development of stratified epithelia, such as the epidermis. To determine the role of p63 isoforms during epidermal development, we are using *in vitro* and *in vivo* models. We demonstrated that two p63 isoforms that contain a transactivation domain, TAp63α and TAp63γ, can induce K14 expression in cell lines and primary $p63^{-/-}$ keratinocytes. Since K14 is one of the first structural proteins expressed during normal epidermal development, this suggests a role for TA isoforms in the commitment of single-layered epithelia to squamous epithelial lineages. To determine the *in vivo* functions of TAp63α and ΔNp63α in epidermal differentiation, we are using inducible mouse models in which epidermal-specific transgene expression is achieved by topical application of RU486. Inducible TAp63α and ΔNp63α mice were bred with mice carrying the activator construct driven by a K14 promoter (K14.Glp65). Skin biopsies from RU486-treated newborn or adult mice exhibited severe hyperplasia in K14.Glp65/TAp63α skin while K14.Glp65/ΔNp63α skin was mildly hyperplastic. In addition, the differentiation process in K14.Glp65/TAp63α skin was perturbed as demonstrated by a delayed onset of K1 expression. Despite the mild hyperplasia, K1 expression appeared normal in K14.Glp65/ΔNp63α skin. In addition, more BrdU-positive cells were observed in the epidermis of K14.Glp65/TAp63α as compared to K14.Glp65/ΔNp63α mice. In summary, transient expression of TAp63α in the epidermis results in perturbed epidermal differentiation, hyperproliferation and severe hyperplasia. Expression of ΔNp63α, however, results in mild hyperplasia without effects on epidermal differentiation. These data suggest that although TAp63α is required for the induction of a squamous differentiation program, it prevents terminal differentiation. Therefore, downregulation of TAp63α and induction of other p63 isoforms may be required for normal epidermal differentiation.

0811

Identification of androgen-inducible TGF-beta1 from balding dermal papilla cells as a key mediator in pathogenesis of androgenetic alopecia

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We attempted establishing an in vitro coculture system by using human dermal papilla cells (DPCs) from androgenetic alopecia (AGA) and keratinocytes (KCs) to explore the role of androgens in hair growth regulation. Androgen showed no significant effect on the growth of KCs when they were cocultured with DPCs from AGA. Because the expressions of mRNA of androgen receptor (AR) decreased during subcultivation of DPCs in vitro, we transiently transfected the AR expression vector into the DPCs and cocultured them with KCs. In this modified coculture, androgen significantly suppressed the growth of KCs by ~50%, indicating that overexpression of AR can restore the responsiveness of the DPCs to androgen in vivo. We found that androgen stimulated the expression of TGF-beta1 mRNA in the cocultured DPCs. ELISA assays demonstrated that androgen treatment increased the secretion of both total and active TGF-beta1 in the conditioned medium. Moreover, the neutralizing anti-TGF-beta1 antibody reversed the androgen-elicited growth inhibition of KCs in a dose-dependent manner. Together, androgen-inducible TGF-beta1 derived from DPCs of AGA is involved in epithelial cell growth suppression in our coculture system, suggesting a potential role of TGF-beta1 in the pathogenesis of AGA.

0812

Localization of versican in human hair follicle: implication of proteoglycan involvement in anagen induction and hair elongation

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Proteoglycans are present in the extracellular matrix and play multiple roles in regulating cell-cell communication and modulating growth factor activity and turnover. Versican is a large chondroitin sulphate proteoglycan and transgenic mouse study showed its pivotal role both in mesenchymal condensation and hair induction. Versican immunoreactivity has been localized in the dermal papilla (DP) of active hair follicles (anagen) in mice but not clear in human species. We examined versican mRNA expression in human hair follicles using the automated in situ hybridization (ISH) system and also compared the sites of versican mRNA expression with the sites of versican immunoreactivity. Human scalp skin specimens (29 years old male and 50 years old male) obtained from plastic surgery were fixed with 10 % phosphate-buffered formalin and riboprobes specific to human versican were prepared by in vitro transcription. The 2-B-1 monoclonal antibody against versican core protein moiety was used for the immunohistochemical (IHC). Versican mRNA was expressed in the DP cells in anagen hair follicles, but not in dermis. Expression of versican gene was apparently decreased in the DP cells of catagen hair follicles and was not detectable in telogen hair follicles. Consistent with ISH results, versican immunoreactivity was extended over the DP of anagen hair follicles. This staining was diminished in the catagen phase of human hair follicles. The remaining activity of the versican signals at catagen stage confined at the upper side of DP by ISH and IHC.. Versican immunoreactivity in the DP was partially lost in terminal hair follicles and almost lost in vellus hair follicles seen in the tissue sections of male pattern baldness. Interestingly, versican immunoreactivity was also remained around the bulge region of anagen hair follicle although no mRNA expression was detected. Our result suggests that versican production may be important for hair induction and maintenance of the normal growing phase of human hair follicles.

0813

Primary cicatricial alopecia: histopathologic findings do not distinguish clinical variants

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All patients with active primary cicatricial alopecia seen at the UCSF Dermatology clinic were eligible. Patients with active disease had symptoms of itching, burning, or pain, had progression of hair loss, a positive pull test, and clinical evidence of inflammation. Two 4 mm scalp biopsies (affected and unaffected) were done. 6 horizontal sections were cut and stained with hematoxylin and eosin and interpreted blindly by one investigator (JTH). 20 patients (5 male/15 female) were enrolled with an average age of 48 years and average duration of disease of 7 years. Clinical diagnosis included: lichen planopilaris (LPP)(5), frontal fibrosing alopecia(2), pseudopelade(Brocq)(3), central centrifugal alopecia(2), folliculitis decalvans(6), and tufted folliculitis(2). Histologic and clinical diagnoses were concordant in 14/20 cases(70%). Biopsy samples of clinically uninvolved scalp had evidence of disease in 11/20 patients (55%). Sebaceous glands were absent in 19/20 (95%) of the biopsies from clinically affected lesions. Tufted folliculitis was histologically indistinguishable from folliculitis decalvans, and frontal fibrosing alopecia was indistinguishable from LPP. In addition, biopsies from LPP and frontal fibrosing alopecia were indistinguishable from other lymphocyte-mediated cicatricial alopecias including pseudopelade (Brocq) and central centrifugal alopecia. We therefore conclude that current histopathologic techinques do not distinguish clinical variants of the above primary cicatricial alopecias. A descriptive, standardized histopathologic reporting of follicular architecture, type, location, and extent of the inflammatory infiltrate, presence or absence of sebaceous glands, presence of fibrosis, may be of most benefit to clinicians evaluating and treating patients with primary cicatricial alopecia.

0814

Eflornithine hydrochloride 13.9% cream alters beard hair fiber curvature and diameter in African-American men with pseudofolliculitis barbae

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This 16 week, open-label investigation evaluated the effects of eflornithine hydrochloride 13.9% topical cream on ten African-American men with a two-year history of at least Grade 3 seudofolliculitis barbae (PFB). Eflornithine hydrochloride 13.9% cream, an irreversible inhibitor of ornithine decarboxylase, has been shown to inhibit the organization of cytoplasmic actin filaments in cultured keratinocytes. We hypothesized that application of eflornithine hydrochloride 13.9% cream would alter the actin cytoskeleton of the beard hair follicle, cause a loss of follicle integrity, and affect hair fiber development. A total of 1,246 pre- and post-treatment beard hair fibers were collected and stained with rhodamine for analysis. Curvature, diameter, and cuticle scale structure and patterns were determined using image analysis and laser scanning confocal microscopy. The PFB clinical rating improved in 8 of the 10 subjects treated with eflornithine hydrochloride 13.9% cream. There were no consistent changes in the cortical, medullary or cuticular structures or cuticle scale patterns. However, post-treatment beard hair fibers had statistically significant higher fiber diameters than pre-treatment fibers (135.8 microns vs. 130.7 microns, p=0.037). An increased proportion of the post-treatment beard hair fibers also had distorted cross-section morphology compared to the circular axis of the pre-treatment fibers. No significant differences in fiber curvature were observed between the pre- and post-treatment groups of hair fibers. However, 6 of 10 patients demonstrated statistically significant changes of fiber curvature. Changes observed in erupted beard hair fiber curvature and diameter after treatment with eflornithine hydrochloride 13.9% topical cream most likely resulted from disruption of the actin cytoskeleton surrounding the beard hair follicle.

0815

Epidermal hyperplasia and impaired morphogenesis of hair follicles in mice overexpressing a soluble form of heparin-binding EGF-like growth factor

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HB-EGF, an EGF receptor ligand, is initially synthesized as a membrane-anchored form (proHB-EGF), which is cleaved to produce a soluble form (sHB-EGF). The involvement of HB-EGF in keratinocyte growth and migration has been well studied. However, the roles of HB-EGF and its processing in skin development remain unclear. Therefore, we generated mice overexpressing sHB-EGF under control of the K5 promoter and investigated its roles in skin development. The transgenic mice showed epidermal hyperplasia and loss of hair. Histological analyses of the newborn mice indicated impaired morphogenesis of follicular dermal papilla in skin overexpressing sHB-EGF. In the embryonic period, mesenchymal aggregates under the hair placode, a precursor of the follicular dermal papilla, make a tightly packed population of cells with little extracellular space. After cell "compaction", the cells separate and the extracellular space is restored in the dermal papilla after birth. At postnatal day 1 (P1), the expression of heparan sulfate proteoglycan (HSPG), an extracellular matrix molecule, was observed and the cells separated from each other in the dermal papilla of wild-type mice. In the transgenic mice, however, HSPG was not detected and the cells tightly adhered to one another in the dermal papilla of P1 skin, indicating that the dermal papillae of transgenic mice remain in the compacted stage; subsequent development of the papillae and hair follicles does not occur. The EGF receptor was detected in epidermal, but not the papilla, cells. These results suggest that regulated processing of proHB-EGF is required for the organization of epidermal tissues and dermal papilla formation in direct and indirect manners, respectively.

0816

Localization of Myc/Mad1 protein in the hair follicle during hair cycle

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Precise molecular mechanisms associated with transient growth/differentiation of follicular keratinocyte have not been fully elucidated in the hair cycle. Resent observations have suggested that proto-oncogene c-myc plays a pivotal role in the regulation of both apoptosis and growth in the epithelial cells. Mad1 is a Myc antagonist that heterodimerizes with Max and functions as a transcriptional repressor. Inhibition of proliferative activity subsequent to Mad1 overexpression has been demonstrated in variety of cell types, including keratinocytes. However, little is known about the Myc/Mad1 expression in follicular keratinocyte. (a) To elucidate whether c-Myc/Mad1 proto-oncogenes could be involved in hair cycle, in situ expression of Myc/Mad1 protein was investigated. (b) Immunohistochemical analysis were performed for the specimens taken from the plucking-induced anagen hair follicle in C3H/HeNCrj mice and human anagen follicle. Antibodies against PCNA (proliferating cell nuclear antigen), c-Myc, Mad1 were used. The expression of c-myc/mad1 mRNA were semi-quantitated in different phases of hair cycle by RT-PCR. (c) Immunoreactivity for PCNA was intensely observed both in the middle portion and matrix of hair folicle in the early anagen of plucking-induced hair regrowth in C3H mice. In mid anagen phase, PCNA positive cells were lacated only the matrix. The expression of c-myc mRNA was detected in the mid-anagen phase, whereas mad1 mRNA was expressed in the early anagen phase. In contrast, c-Myc-positive cells were visible in Huxley's and Henley's layer of inner root sheath and Mad1 was expressed in whole hair follicle by confocal microscope. (d) These suggested that cells in mid-portion of hair follicle proliferate transiently before plucking-induced anagen phase. Moreover, c-myc and mad overexpression might be related with switching to the hair cycle. These findngs suggested that c-myc has been implicated not only in the cell proliferation, but also in the terminal differentiation and mad has been implicated in the cell differentiation in hair cycle

0817

Post-natal acidification of stratum corneum in the neonate: functional consequences for the neonate of a neutral pH

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At birth, mammalian stratum corneum (SC) displays a near-neutral surface pH, which declines over several days to weeks to an acidic pH, comparable to adults. An acidic pH is required for permeability barrier homeostasis and SC integrity/cohesion. The post-natal acidification in the neonatal rat SC where pH, as measured with a flat surface electrode, declines progressively from near-neutral levels (pH 6.8) on day 0-1 post-natal to adult levels (pH 5.6) over the subsequent 5-6 days. The basal transepidermal water loss (TEWL) is normal while acute barrier disruption by both tape stripping and acetone treatment shows a delay in barrier recovery at day 0/1 compared to day 5/6. Acidification experiments with "super-acids" and acid buffer systems reversed the negative impact of the near-neutral pH at birth on permeability barrier homeostasis. On ruthenium tetroxide-stained electron-microscopy, the delay in barrier recovery was paralleled by normal lamellar body secretion, but evidence of abnormal (=delayed) lamellar bilayer maturation. The biochemical basis for delayed maturation could be further attributed to decreased beta-glucocerebrosidase activity. Blockade of the sPLA2 and NHE1 pathways, previously shown to contribute to formation of an acid SC pH, provoked a delay in neonatal acidification, and resulted in an impaired barrier homeostasis. Furthermore SC integrity/cohesion were impaired in either the case of a near-neutral pH at day 0/1 or following blockade of the sPLA2 and NHE1 pathways. The impaired SC integrity/cohesion was associated with a decrease in corneodesmosomes and in desmoglein 1. Together, these results demonstrate the progressively beneficial effect of the post-natal development of an acidic pH on permeability barrier homeostasis and SC integrity/cohesion

0818

Effect of transforming growth factor-b on the proliferation and differentiation of multipotential sphere colonies from skin

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Skin derived precursor cells possess self-renewal and multipotential capabilities, which can give rise to neuron, glia, smooth muscle cells (SMC) and adipocyte. Previous studies in vitro and in vivo have demonstrated that transforming growth factor-b (TGF-b) plays a pivotal role in controlling proliferation and differentiation of the skin cells. In this study, we investigated the effects of TGF-b on mutipotential sphere colonies from the ear skin using a sphere forming assay, in which the multipotential cells can be grown in response to epidermal growth factor and basic fibroblast growth factor to form floating clusters of cells called sphere colonies. We found that the number, but not the diameter, of the sphere colonies increased with an addition of TGF-b during the colony formation. However, the capabilities of differentiation of the sphere colonies generated in the presence of TGF-b were unaffected, as analyzed by the immunostaining of the differentiated progenies with antibodies directed for neuron (b3 tubulin and neurofilament M), astroglia (Glial Fibrillary Acidic Protein) and SMCs (a smooth muscle actin) together with an Oil red O lipid droplet staining. Similar results were obtained when TGF-b was added after the cells were passaged, i.e. the number, but not the diameter, of the daughter sphere colonies increased without affecting their capabilities of differentiation. Interestingly, the percentage of SMCs increased when TGF-b was added after differentiation was initiated, whereas the differentiation into other cell types was not perturbed. Together our results demonstrate that the addition of TGF-b increased the number of the cells to initiate sphere colony formation, raising the possibility that TGF-b can induce transdifferentiation of the skin cells into immature phenotypes and the effect of TGF-b changes once a cue for differentiation is given.

0819

Role of prolactin in human hair growth control: cross-connections to androgen receptor signaling?

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Murine skin expresses prolactin (PRL), and PRL treatment induces premature catagen in murine skin organ culture (Foitzik et al., Am J Pathol, in press), while PRL receptor (PRLR) null mice show an altered hair follicle cycling pattern (Craven et al., Endocrinology 142:2533, 2001). Here, we have investigated the expression of PRL and PRLR in human hair follicles (HF), and have studied the influence of PRL on isolated human hair follicles in anagen VI. By immunohistochemistry (IH), prolactin immunoreactivity (IR) was detected in human anagen VI HF in follicular keratinocytes, while the dermal papilla was negative. Prolactin receptor IR showed a very similar pattern. In catagen HF, prolactin and PRL receptor expression appeared to be upregulated. Contrary to a previous report that PRL mRNA is not transcribed in human skin (Slominski et al., J Lab Clin Med 136:476, 2000), we show by RT-PCR that human hair follicles do express the PRL gene. Prl (400 ng/ml) significantly inhibited hair shaft elongation and accelerated a catagen-like HF transformation in isolated human hair follicles, where it also significantly reduced the number of proliferating and increased the number of apoptotic cells in the hair bulb. When visualizing androgen receptors by IH, PRL-treated hair follicles showed a substantial up-regulation of androgen receptor-IR in the hair bulb. Currently, we are testing whether co-stimulation of human hair follicles with PRL and androgen receptor antagonists abrogates the inhibitory influences on human hair growth in vitro, are searching for target genes of PRLR-mediated signaling in the hair follicle and are investigating the influence of PRL on follicular androgen metabolism. These data support the hypothesis that PRL is a locally generated hair growth inhibitor, possibly not only via direct, PRLR-mediated effects but also via upregulation of androgen receptors.

0820

Expression and localisation of tight junction proteins in human hair follicles

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Tight Junctions (TJ) are intercellular junctions selectively sealing cells and controlling the transcellular pathway. They are well characterized in simple epithelia and endothelia but have only recently been described in stratified epithelia such as epidermis. The human hair follicle comprises various epithelial layers which are partly in morphogenical continuity with the epidermis and develop there from during early fetal life. The barrier function of these epithelial layers seems to be important for the universal continuity of the barrier represented by the skin. By using RT-PCR-techniques and immunolocalization we show the presence of the TJ proteins ZO-1, occludin, and various claudins in the hair follicle and demonstrate their heterogeneous distribution pattern within a given strata as well as within the different epithelial layers. A colocalization of the various TJ proteins, arguing for typical tight junction structures, can be observed especially in cell layers facing the hair shaft and the stratum corneum, and in addition at the border between outer and inner root sheaths. Usually they are found in close vicinity to desmosomal and adherens junction proteins. The morphological and biological importance of these findings and the possible roles of TJ in hair follicles, e.g. in follicular penetration and prevention of transepidermal water loss, are discussed.

0821

17beta-estradiol, but not 17alpha-estradiol inhibits human hair growth in whole follicle organ culture

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It has been know for a number of years that ovarian hormones can significantly influence the hair cycle. In rats, mice and guinea pigs it has been clearly demonstrated that estrogens inhibit hair growth. Although we have recently reported that estrogen receptors are expressed in the human hair follicle, the role of estrogens in human hair growth is still unclear. Therefore, we have used a whole follicle organ culture system to investigate the effect of both 17beta and 17alpha-estradiol on human hair growth. Individual hair follicles from female occipital scalp were microdissected out from surrounding skin and incubated individually in phenol red-free William's E medium supplemented with L-glutamine, penicillin, streptomycin, 5mM glucose and either 10nM 17beta-estradiol, 10nM 17alpha-estradiol or the vehicle control (0.0001% alcohol) (6 follicles per group). The increase in the length of the follicles was measured sequentially with an inverted microscope fitted with an eyepiece measuring graticule over a seven day period. Follicles in each group exhibited linear growth over the seven day period. Follicles incubated with 17alpha-estradiol grew at the same rate as the control group. However, follicles incubated with 17beta-estradiol grew at a significantly slower rate than the control group ($p<0.0001$). These reults show that the biologically active estrogen, 17beta-estradiol, has a physiological effect on human hair growth in culture, which parallels the *in vivo* effects seen in animals. We have previously shown that ERbeta is the receptor expressed in human hair follicles in situ and that it is expressed in the cells of the dermal papilla and follicular epithelium. Studies are in progress to further elucidate the mechanism of action of estrogens and estrogen receptors in the human hair follicle.

0822

Epidermal and hair follicle progenitor cells express melanoma-associated chondroitin sulphate proteoglycan

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Basal keratinocytes in the epidermis and hair follicle are biologically heterogenous but must include a stable subpopulation of epidermal stem cells. In animal models these can be identifed by their retention of radioactive label due to their slow cycle (label retaining cells) but human studies largely depend on in vitro characterisation of colony forming efficiency and clonogenicity. Differential integrin expression has been used to detect cells of increased proliferaitive potential but further stem cell markers are urgently required for in vivo and in vitro characterisation. Using LHM2, a monoclonal antibody reacting with a high molecular weight melanoma associated proteoglycan (MCSP) a subset of basal keratinocytes in both the interfollicular epidermis and the hair follicle have been identified. Co-expression of MCSP with keratins K15 and K19 as well as b1 and a6 integrins have been examined in adult and foetal human skin from hair bearing, non-hair bearing and palmoplantar regions. Although MCSP co-expression with a subset of b1 integrin bright basal keratinocytes within the epidermis would suggest that MCSP co-localises with epidermal stem cells, MCSP expression within the hair follicle was more complex and multiple sub-populations of basal ORS keratinocytes are described. These data suggest that epithelial compartmentalisation of the ORS is more complex than interfollicular epidermis and further supports the hypothesis that more than one hair follicle stem cell compartment may exist.

0823

The cutaneous vanilloid receptor 1 expression suggests multiple functions beyond sensory nerve signaling

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Vanilloid receptor 1 (VR1), a key molecular integrator of nociceptive sensory mechanisms, was recently described to be functionally expressed in human epidermal keratinocytes. In this study, using functional assays and immunohistochemical methods, our goal was to describe the expression of VR1 in other cell types of human and mouse skin preparations. Both in mouse and human skin, VR1 immunoreactivity (ir) was localized to the (mostly superficial layers of the) epidermis and to inner and outer root sheath keratinocytes (but not to the dermal papilla fibroblasts) of hair follicles. Furthermore, in human skin but not in mouse skin, strong VR1-ir was observed in sweat and sebaceous glands, in smooth muscle cells of vessels, and, as was revealed by double staining with cell-specific markers, in mast cells and dendritic cells. Since hair follicles provide an excellent model for exploring actions of various agents on the proliferation and differentiation of defined epithelial cell subpopulations, we also investigated the effects of VR1 acting drugs on hair growth characteristics. Preliminary results suggest that modification of VR1 by various vanilloid compounds (the agonist capsaicin and the antagonist capsazepine) modulated hair shaft elongation of organ-cultured human scalp hair follicles in a dose-dependent and VR1 receptor-mediated fashion. In summary, VR1 is expressed in various cutaneous cell populations, and the functional presence of this receptor/channel in hair follicle keratinocytes suggests that VR1-mediated signaling is involved in the regulation of follicular epithelial cell growth and differentiation.

0824

Estrogen effects on murine and human hair follicle cycling

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Although 17 β-estradiol (E2) is a potent hair growth modulator, our knowledge of estrogen effects on hair follicle cycling is still very limited. Here, we have investigated hair cycle-dependent estrogen receptor (ER) expression patterns, how E2 affects murine catagen development, and whether E2 influences development and recovery from chemotherapy-induced alopecia in mice. Immunohistochemical staining (ABC-DAB, TSA) for ER α and ER β revealed that both receptor subtypes are expressed throughout the murine hair cycle, with a maximum in telogen dermal papillae and sebaceous gland. By RT-PCR we detected ER α, but not ER β transcripts during the entire murine hair cycle, with a maximum in catagen and telogen skin. Female C57BL/6 mice were treated topically with E2 or with an ER antagonist (ICI 182.780) when follicles were in anagen stage VI. Staging was assessed macroscopically by dotmatrix planimetry and by quantitative histomorphometry. We demonstrate that topical E2 induces premature catagen development while the ER antagonist had no effects on catagen development. By promoting the dystrophic catagen pathway, topical E2 enhanced cyclophosphamide-induced alopecia and significantly stimulated the re-growth of normally pigmented hair shafts. In organ-cultured human scalp hair follicles (female, temporal region), E2 (10-6-10-9 M) slightly, but non-significantly, inhibited human hair shaft elongation *in vitro* and rather prolonged the duration of the anagen phase *in vitro* (10-7 M). We conclude that murine pelage hair follicles (fur coat) *in vivo* appear to show opposite E2 hair cycling effects compared with human scalp hair follicles *in vitro*.

0825

Withdrawn.

0826

Fas and Fas-ligand are involved in the control of hair follicle response to chemotherapy

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Anticancer drugs induce apoptosis in the hair follicles and hair loss, the most common side effect of chemotherapy. Hair follicle response to cyclophosphamide is p53-dependent, and p53 mediates chemotherapy-induced apoptosis in the hair follicle via modulating the transcription of p53 target genes. In a C57BL/6 mouse model for chemotherapy-induced hair loss, we demonstrate that p53 targets Fas and Fas-ligand are involved in the control of hair follicle response to chemotherapy. By semi-quantitative RT-PCR, the expression of both Fas and Fas-ligand transcripts is increased in full thickness skin samples 24-72 hours after cyclophosphamide administration. By immunohistology, Fas and Fas ligand are upregulated and co-localized with TUNEL in the hair matrix, outer and inner root sheaths after cyclophosphamide treatment. In contrast to wild type mice, Fas-deficient mice show significantly ($p<0.05$) reduced apoptosis in the hair follicle keratinocytes and retardation of cyclophosphamide-induced hair follicle regression. Furthermore, administration of Fas-ligand neutralizing antibody into wild type mice significantly ($p<0.05$) reduced a number of apoptotic cells in the hair follicle and retarded a rate of hair follicle involution induced by cyclophosphamide. This suggests that Fas signaling pathway is involved in apoptosis of hair follicle keratinocytes induced by cyclophosphamide. These observations also indicate that locally administered inhibitors of Fas signaling may be combined with p53 inhibitors to prevent or retard hair follicle response to chemotherapy and chemotherapy-associated hair loss.

0827

Scalp dermal papilla cell conditioned medium inhibits DMEM induced ^{3}H-thymidine uptake by epidermal keratinocytes and is modulated by estradiol and tamoxifen

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Estrogens influence the hair cycle, yet the cellular sites and mechanisms of estrogen action are still poorly understood. Radioactive estrogens and androgens have been localised to the follicular dermal papilla in rodents and recently we reported that estrogen receptor beta is expressed in human hair follicles in situ and dermal papilla cells *in vitro*. Since we have previously demonstrated androgens stimulate cultured dermal papilla cells to secrete autocrine growth factors, we have incubated cultured dermal papilla cells with estrogenic and non-estrogenic steroids to investigate the effect of conditioned media on the DNA synthesis of primary cultures of epidermal keratinocytes. Primary cultures of dermal papilla cells and epidermal keratinocytes were propagated using established methods. Primary dermal papilla cell lines (n=3) were incubated in phenol-free, serum-free DMEM in the presence of vehicle control (0.0001% alcohol), 10nM 17β-estradiol, 10nM estrone, 10nM testosterone, 10nM DHT, 10nM 5α-androstane-3α, 17β-diol, 10nM 5α-androstane-3β, 17β-diol, 100nM dexamethasone, 100nM tamoxifen or tamoxifen plus 17β-estradiol for 24h. The media was collected and its effect on keratinocytes assayed using ^{3}H-thymidine. Parallel dishes of keratinocytes were incubated with unconditioned DMEM or keratinocyte serum-free medium +/- the relevant steroids. Unconditioned DMEM significantly increased DNA synthesis by keratinocytes compared to control keratinocyte serum-free medium ($p<0.0001$). In contrast dermal papilla cell conditioned DMEM significantly inhibited this response ($p<0.0001$). Furthermore, 17β-estradiol augmented this inhibition, which was antagonised by tamoxifen, while estradiol attenuated the stimulatory effect of tamoxifen. These results further amplify the role of dermal papilla cells in the regulation of epithelial cells and provide important evidence for a role for estrogenic steroids in this process.

0828

Multiple functions of Noggin in cutaneous development: leads and lessons from Noggin transgenic mice

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Noggin is an antagonist of bone morphogenetic proteins 2/4 (BMP-2/4), which play pivotal roles in regulation cell proliferation, differentiation and apoptosis during development. To study the in vivo functions of noggin in cutaneous development, transgenic mice overexpressing noggin under control of the K5 promoter were generated using C3H/HeJ mice as a background strain. K5-Noggin transgenic mice are viable and fertile and show several cutaneous phenotypes different to the wild type (WT) controls. Firstly, K5-Noggin mice show acceleration of hair follicle morphogenesis and formation of enlarged hair follicles with increased proliferative activity in the hair bulb. Secondly, K5-Noggin mice show hyperpigmented hairs with predominance of eumelanin, while agouti-type hair pigmentation is seen in WT mice. Thirdly, K5-Noggin mice show severe alterations of keratinocyte apoptosis and differentiation in the eyelids, which results in retardation of the eyelid opening by 17-24 days. Reduction of apoptosis and retardation of cell differentiation seen in the eyelid epithelium of K5-Noggin mice are associated with downregulation of expression of the apoptotic receptors (Fas, p55 kD TNFR), Id3 protein, and keratinocyte differentiation markers (loricrin, involucrin). Since BMP-4, but not EGF accelerates opening of the eyelid explants isolated from K5-Noggin transgenic mice and cultured *ex vivo*, this suggests that BMP signaling pathway operate independently of EGF signaling in controlling eyelid opening. Thus, BMP-2/4 and noggin function as critically important players in the control of genetic program of cutaneous development. Most importantly, noggin and BMP-2/4 regulate not only morphogenesis of skin appendages, but also control the formation of openings in skin overlying sensory organs, such as the eye, to provide access for environmental signals to sensory epithelia.

0829

CD34 expression delineates a population of outer root sheath keratinocytes outside the bulge area in the lower human anagen hair follicle

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Hair follicle bulge cells have properties of epithelial stem cells in that they are highly persistent, rarely proliferate and can give rise to multiple cutaneous epithelial cell types in vivo. Recently, we described CD34 as a cell surface marker for bulge cells in the mouse. To study the relevance of CD34 expression in human hair follicle stem cell biology, we immunostained tissue sections of human scalp for CD34. Using two different anti-CD34 antibodies, we found that, unlike CD34 expression in the mouse, CD34 was not expressed in human hair follicle bulge cells of telogen or anagen hair follicles. Rather, within the cutaneous epithelium, CD34 expression was confined only to anagen hair follicles, in the keratinocytes of the basal outer root sheath below the bulge and above the bulb. The morphology of these cells differs from the bulge cells in that they are columnar and heavily glycogenated. Based on bromodeoxyuridine incorporation of human skin grafted to immunodeficient mice, these cells proliferate more frequently than the bulge cells but less frequently than the bulb matrix cells. Thus, CD34 expression defines outer root sheath cells within the human anagen hair follicle that appear to have proliferative characteristics of early transit amplifying cells. Furthermore, although bulge cells within both mouse and human preferentially express cytokeratin 15, their differing expression patterns with respect to CD34 may reflect the extremely different anagen durations of mouse and human hair follicles.

0830

Effect of Aldara 5% cream on anagen:telogen and terminal:vellus ratios in extensive alopecia areata

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In this study we asked if daily application of Aldara 5% Cream (3M Pharmaceuticals) to 1/2 of the scalp in 12 Caucasian patients with extensive alopecia areata (AA), (5M, 7F, ages 30-68, average disease duration 14 years)induces hair growth. Two 4-mm punch biopsy specimens were taken from the right parietal scalp before and after treatment. 11 patients successfully completed the study. Scalp biopsy specimens were vertically and horizontally sectioned, stained using standard techniques, and analyzed by a dermatopathologist (DW) who was blinded to patient information. Anagen:telogen ratios, terminal:vellus ratios, follicular structures per mm2 and inflammation were assessed. No significant hair regrowth was observed; however, the number of follicular structures per mm2 increased in 4/11 subjects; terminal:vellus ratios increased in 9/11 subjects, and anagen:telogen ratios increased in 7/11 subjects. Lymphohistiocytic infiltrates were noted to decrease in 3/11 subjects, increase in 3/11 subjects and remain the same in 5/11 subjects. Aldara 5% cream augments interleukin-2 production and enhances Th1 responses in the skin, changes which have been implicated in the pathophysiology of AA. So not unexpectedly, no patient with extensive AA demonstrated a significant clinical improvement after only 6 months of therapy. However, the surprising outcome of this study was that the use of Aldara 5% cream did affect the hair follicle and hair cycle as noted in the skin biopsy specimens. It is possible this immunomodulating agent may prove to have a role in the management of extensive AA if the treatment period is longer and there is histological potential for hair regrowth at baseline.

0831

Epidermal-specific loss of BMP receptor 1A results in dysgenesis of hair and teeth

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Several lines of evidence implicate BMP signaling in the development of epidermal appendages such as hair and teeth. However, early lethality has precluded analysis of the effects of loss of function mutations in BMP pathway genes on hair and tooth development. Here we have used conditional gene targeting to reveal roles for the most widely expressed type I BMP receptor, BMPR-IA, in hair and tooth development. We utilized several different lines of transgenic mice that express Cre recombinase in surface ectoderm: Brn4-Cre pedigree bcre-32, that shows ectopic Cre expression in limb and mid-ventral ectoderm from 9.75 days post-coitum; and two mouse lines in which Cre is placed under the control of a keratin 14 (K14) promoter that is active in surface ectoderm from embryonic day 9.5 and subsequently in the basal layer of the epidermis, hair follicles and tooth epithelium. We found that mice that are homozygous for a conditional allele of the Bmpr1a gene (Bmpr1a-cl) and also carry bcre-32 survive up to 14-20 days after birth and display limb defects and a hairless midventrum. Bmpr1a-cl/Bmpr1a-cl mice carrying a highly active K14-Cre transgene die at birth with limb defects and open eyes. However, use of a second, less active K14-Cre transgenic line prevented perinatal lethality. These mice displayed missing or defective incisor teeth and extremely sparse, short hair. Analysis of the histology of newborn and postnatal skin from bcre-32; Bmpr1a-cl/Bmpr1a-cl and K14-Cre; Bmpr1a-cl/Bmpr1a-cl mice revealed an apparently normal density and spacing of hair follicles in each case. However, differentiation of the hair shaft was severely disrupted in midventral skin from bcre-32; Bmpr1a-cl/Bmpr1a-cl mice and in ventral and dorsal skin in K14-Cre; Bmpr1a-cl/Bmpr1a-cl mice. These results indicate that signaling through epithelial BMPR-1A is not required for the initial stages of hair follicle morphogenesis, but plays an essential role in hair shaft differentiation and in tooth development.

0832

Hair growth cycle modulation of the β-endorphin/μ-opiate receptor system in human follicular melanocytes

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The opiate peptide b-endorphin (b-END) is enzymatically cleaved from POMC in the skin. Cutaneous functions of this peptide include immunomodulation and melanogenesis, effects that are mediated via opiate receptors. Unlike other POMC peptides, a role for b-END in hair pigmentation/cycling has not been demonstrated. This study was designed to assess expression of the b-END/mu-opiate receptor system in human hair follicle melanocytes (HFM) during the hair growth cycle. Anagen VI was associated with a striking up-regulation of b-END expression in both melanogenically-active bulbar melanocytes and amelanotic outer root sheath (ORS) melanocytes. b-END expression was also marked in the epithelial compartments but was conspicuously absent in the follicular papilla. Catagen however, was associated with down-regulation in b-END expression in residual gp-100 positive melanocytes, while strongly b-END-positive melanocytes were found in the peripheral regions of the epithelial sac in late catagen, early telogen, and late telogen follicles. The mu-opiate receptor also showed hair cycle-dependent change, whereby this receptor during anagen was detected only in a sub-population of weakly melanogenic melanocytes both in the proximal/peripheral hair bulb matrix and of amelanotic melanocytes in the ORS. In catagen and telogen melanocyte expression of mu-opiate receptor was moderately down-regulated. Receptor expression was detected in rare non-dendritic melanocytes located in the residual catagen bulb and also in melanocytes located in the peripheral telogen sac. The follicular papilla and hair follicle epithelium showed prominent mu-opiate receptor expression throughout the cycle. The differential expression of the b-END/ mu-opiate receptor system in follicular melanocytes during the hair growth cycle, and the up-regulation of b-END expression in differentiated bulbar melanocytes, suggests an important role for this opiate in maintaining melanogenesis during hair growth.

0833

Signal transduction pathways responsible for restoring hair growth in alopecia areata mice

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It has been found that mechlorethamine is the most effective therapy for alopecia areata (AA)-affected C3H/HeJ mice and this is an ideal model system to explore the corresponding molecular mechanisms of drug's therapeutic actions on reversing autoimmune arrested follicular activity in AA. The effects of various immuno-modulating drugs have been studied for different autoimmune disorders, but little is known about the signaling pathways involved in their therapeutic responses. To study the signaling pathways responsible for successful mechlorethamine therapy in AA mouse model, a Western blotting-based multi-antibody proteomics assay was used. A total of 52 different protein kinases were detected in the C3H/HeJ mouse skin and over 17 protein kinases showed significant differences between control and mechlorethamine-treated skins. Protein kinases in the growth factor-mediated Erk pathway (Raf1, Mek1, Erk1/2/3, S6K), the cell cycle regulatory kinases (CDK4/5/6/7), and the multi-functional GSK-3 kinases were increased by mechlorethamine. The later have been linked to follicular activity and also inhibit IL-2 production by T cells. Another interesting finding is that the expression of cell adhesion mediated kinase FAK was dramatically decreased by mechlorethamine, suggesting that this kinase might be responsible for T lymphocytes migration into targeted hair follicles. Other kinases modulated by mechlorethamine are either important regulators of cell proliferation/differentiation (PKG, PKB, PKC, CK-2) or linked to immune functions (PIM1, PKB). These signal transduction pathways may represent the initial molecular events in mouse skin upon successful mechlorethamine treatment and will accelerate the target discovery process for treating AA. The findings from this study may also shed light on the precise mechanism of action of mechlorethamine on cutaneous T cell lymphoma.

0834

Nestin-expressing hair follicle stem cells

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The nestin gene encodes an intermediate filament protein which is selectively present in the neuroepithelial cells of the developing embryo and in the neurogenic areas of the adult brain. Transgenic mice, in which nestin regulatory elements drive expression of green fluorescent protein (GFP) were found to have nestin-expressing cells located in the permanent upper hair follicle right below the sebaseous glands in the follicle bulge. This is the area of the follicle where the hair follicle stem cells have been previously shown to be located. The nestin-GFP-expressing stem cells in the hair follicle bulge give rise to the outer root sheath and possibly other structures during anagen. The nestin-GFP cells in the hair follicle cells co-express nestin and keratins as determined by immunohistochemistry. These data indicate the nestin-expressing cells in the bulge area of the follicle are the hair follicle stem cells. Nestin-driven GFP was also found to be highly expressed in an interfollicullar neural-like network. The common expression of nestin in neural stem cells, in hair follicle stem cells, and in an inter-hair-follicle neural-like network suggests their common origin. This gives rise to the possibility of the hair follicle stem cells being able to form neural cells which has many ramifications.

0835

Wnts signaling through the B-catenin pathway maintains anagen characteristics in dermal papilla cells

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Purpose: We have reported previously that treatment of an enriched dermal papilla cell preparation in vitro with Wnts 3a or 7a maintains the ability of these cells to promote hair growth when engrafted with primary keratinocytes in vivo. The effects of these intercellular signaling molecules are mediated by several different signal transduction cascades within the cell. This study was designed to determine which pathway mediates the effects on hair growth promoting activity and to further test approaches to restore that activity to cells that have lost it. Methods: The GFP positive population from versican-GFP; B-actin tva transgenic mouse pups was purified by FACS and infected in vitro with retroviruses encoding Wnts 3a, 7a or truncated forms of B-catenin, or treated with agents that activate the B-catenin pathway. Results: Forced expression of either Wnt in this partially purified dermal population is sufficient to maintain expression of the versican GFP transgene, a surrogate marker for the anagen DP cell state. While Wnt could function in an autocrine or paracrine fashion in this assay, this result confirms that the activity on the GFP expressing cells is direct and not mediated by secondary signaling from another cell type. Activation of the B-catenin pathway directly using the truncated B-catenin construct leads to cell autonomous activation of the reporter. Hence Wnt signaling does not lead to the expression of a second signal from DP cells that maintains gene expression in other Dp cells. Treatment of the cells with LiCl, an inhibitor of GSK-3b that activates the B-catenin pathway, has a similar effect. Discussion: Different reporter assays had raised question whether the B-catenin pathway is normally active in dermal papilla cells in vivo and whether the activity of Wnts observed in vitro on enriched dermal papilla cell preparations is mediated through this pathway. This work confirms that Wnts acting through the B-catenin pathway can maintain gene expression characteristic of the anagen phase in dermal papilla cells.

0836

Sox9 expression in red deer (*Cervus elaphus*) antler tip velvet. A potential role in hair follicle development?

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Sox9 is a Sry type homeobox gene sharing a strongly conserved 79 amino acid high mobility group box (HMG) domain. It has known expression in developing chondrocytes and teeth and also has a role in temperature sensitive sex determination in reptile species not possessing the Sry male sex determining gene. In Man mutations in this gene result in congenital defects with genetic males possessing female phenotypes, as well as skeletal malformations described as campolmelic dysplasia. Mutations in Sox9 are fatal with offspring surviving only a few days after birth. In red deer (*Cervus elaphus*) antlers annually undergo rapid *de novo* development of new bone, skin and hair follicles. We hypothesise that Sox9 is involved in the development of antler cartilage and osseous tissue and that it may also have a role in hair follicle development. With the use of RT-PCR Sox9 mRNA transcripts were identified in all regions of the developing antler tip, which includes the epidermis and dermis, reserve mesenchyme and developing cartilaginous and osseous tissue. Positive control tissue (testes) showed strong expression of this gene as expected. Negative control (heart) tissue did not express Sox9. Results were confirmed in six different deer for the antler velvet epidermis and dermis; other tissues in at least two deer. In addition, expression was also identified in bald velvet showing only very early hair follicle bud developmental stages. The sequenced transcripts showed close similarity with the *Bos taurus* Sox9 gene. The findings in the developing antler tip suggest that Sox9 is involved in bone development with a predicted expression within the developing chondrocytes. In addition, we propose that Sox9 may also have a new and exciting role in the developing epidermis and hair follicles of the antler velvet.

0837

Deregulated c-Myc diverts multipotent stem cells to a sebaceous gland lineage

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c-Myc is an oncoprotein known to induce proliferation, transformation and apoptosis in mammalian cells. To analyze the role of c-Myc *in vivo*, we previously targeted the overexpression of c-Myc to the hair follicles and basal layer of the mouse epidermis. As homozygotes, these mice (K14.myc2) exhibit epidermal hyperplasia at postnatal day 3, are smaller than wild-type littermates, and die before 21 days of age. As adults K14.myc2 heterozygous mice develop spontaneous ulcerated lesions, which shorten their lifespan to 5 months. We have previously documented that this phenotype occurs due to impairment in wound healing and accelerated depletion of epidermal stem cells. These mice also exhibit an increase in the number and size of sebaceous glands and are predisposed to develop sebaceous gland tumors in response to a chemical carcinogenesis protocol. To bypass the premature lethality of K14.myc2 mice, we have generated an inducible mouse model in which c-Myc expression can be regulated by topical application of a progesterone antagonist, RU486. We observed a marked increase in sebaceous gland number in neonates topically treated to induce c-Myc expression, which suggests that c-Myc expression accelerates the rate of sebaceous gland development. Consistent with this data, adult mice show an increase in sebaceous gland number and size after prolonged treatment with RU486. In addition, we are using the inducible system to determine if c-Myc expression can result in precocious sebaceous gland development by inducing c-Myc expression *in utero*. Pregnant females were injected with RU486 and progesterone once a day from embryonic day E14.5 through E17.5. At E19.5, sebaceous glands were difficult to detect in control littermates, however Oil Red O staining revealed accelerated sebaceous gland development in transgenic mice. Taken together, these results suggest that deregulated c-Myc expression may alter the fate of multipotent stem cells, diverting them to a sebaceous gland lineage.

0838

Differential expression of dioxin-binding Ah receptor (AhR) and AhR Nuclear Translocator (ARNT) in mouse skin suggests the role of ARNT-independent pathway in the pathogenesis of chloracne

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The aromatic hydrocarbon receptor (AhR) is a ligand-activated helix-loop-helix transcription factor that belongs to the family of Per-ARNT-Sim proteins and serves as a basic mediator of the toxic effects of the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In the canonical model of dioxin toxicity dimerization of activated AhR with ARNT is a required step. However, recently several ARNT-independent pathways of TCDD toxicity have been identified. The role of AhR and ARNT in skin effects of TCDD action remains unknown. As the first step in elucidation the role of the AhR signaling pathway in chloracne, the temporal and spatial expression patterns of AhR and ARNT1 were studied during hair follicle (HF) development and regression in the C57BL/6 mouse model. During HF morphogenesis, a gradual increase of ARNT1 immunoreactivity was found in the dermal fibroblasts while initially low/moderate expression in HF epithelium, follicular papilla and in the hair matrix gradually declined, and was not recognizable after stages 6-7 of morphogenesis. In contrast to ARNT1, at initial steps of morphogenesis, HF epithelium and epidermis were totally negative for AhR, while strong expression was found in the dermis. At stage 5 of morphogenesis AhR immunoreactivity occurred in melanogenic zone and the lower IRS (Henley's layer), becoming very active by stage 6. With the completion of morphogenesis, AhR immunoreactivity was evident in the epidermis and in the sebaceous gland (SG), and shifted from the Henley's to the Huxley's layer in the IRS. With transition to telogen, expression of AhR in HF declined, remaining at the moderate level only in the SG and epidermis. The weak and transitional expression of ARNT1 in the HF and the lack of spatiotemporal overlap with AhR expression suggest that skin effects of TCDD toxicity may be mediated by AhR via an ARNT-independent mechanism.

0839

Hair cycle regulation of hedgehog signal reception by regulation of Gli protein accumulation

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Proper patterning of self-renewing organs like the hair follicle requires exquisite regulation of growth signals. Sonic hedgehog (Shh) signaling in skin controls the growth and morphogenesis of hair follicle epithelium in part through regulating the Gli transcription factors. While ectopic induction of Shh target genes leads to hair follicle tumors such as basal cell carcinomas, how Shh signaling normally functions during the cyclic process of hair development is unknown. Here we show that, during the hair cycle, Shh expression and the ability of skin cells to respond to Shh signaling is spatially and temporally regulated. Induction of Shh target genes normally occurs only in the anagen hair follicle in response to expression of Shh. However, in patched1 heterozygous mice, putative tumor precursors form with concomitant induction of Shh target gene transcription only during anagen in follicular and interfollicular keratinocytes. Ectopic production of Gli1 accumulates Gli protein and induces Shh target genes and epithelial tumors at anagen but not other stages, pointing to a restricted competence occurring at the level of Gli protein accumulation. Delivery and reception of growth signals amongst multipotent cells are restricted in time and space to facilitate cyclic pattern formation.

0840

Embryonic development of the normal mouse nail unit

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The purpose of this study was to characterize embryonic development of normal mouse nail unit in order to facilitate comparison with the human nail unit and to identify models for abnormal development. Scanning electron microscopy, elemental X-ray microanalysis, histology, and immunohistochemistry were used to define the process. Demarcation of digits was seen at 14.5 days post coitus (dpc). By 15.5 dpc individual digits were distinct. By 16.5 dpc invagination of the proximal nail fold and nail plate formation were noted. Early epidermal differentiation on the embryonic digits could be identified using antibodies directed against specific keratins. Elemental analyses for sulfur concentration in the nail plate (hard keratins, ultrahigh sulfur proteins) compared with skin of the digits (soft keratins, low sulfur containing proteins) revealed that the nail plate does not mature until birth, when sulfur levels dramatically increase. This information is useful for defining developmental steps in the formation of the normal nail unit and for characterization of mutant nail phenotypes.

0841

Evidence for expression of melanocortin receptors, prohormone convertases, pro-opiomelanortin and pro-opiomelanortin-derived peptides in human dermal papilla cells

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Dermal papilla cells (DPC) are crucially involved in regulating the activity of the hair follicle. In contrast to the majority of other skin cells, little is known about the expression, regulation and function of pro-opiomelanocortin (POMC)-derived peptides and their receptors in DPC. We show here that human DPC in vitro express melanocortin-1 and -4 receptors at the RNA and protein level. POMC was detectable at the RNA and protein level as demonstrated by RT-PCR and Western immunoblotting using an anti-ACTH antibody that cross-reacts with POMC. Among various prohormone convertases (PC) examined PC1 and PACE were undetectable while PC2, its cofactor and chaperone 7B2, and furin convertase were expressed in DPC. We next examined the capability of DPC to express and secrete POMC-derived peptides. As shown by radioimmune assays and enzyme-linked immunosorbent assays, both DPC lysates and cell culture supernatants contained immunoreactive ACTH-like peptides as well as beta-endorphin while alpha-MSH was undetectable. Taken together, our findings demonstrate, for the first time, that DPC in culture express a distinct pattern of melanocortin receptors and express ACTH-like peptides as well as endogenous opioids. Processing of POMC into ACTH-like peptides in DPC does not appear to be mediated by PC1 but rather by an alternative POMC cleaving enzyme, e. g. furin convertase. Further studies are necessary to define the exact role of melanocortin receptors and the function of detected POMC peptides in DPC in vitro and in vivo.

0842

Microarray-based gene expression profiling in alopecia areata implicates immune response, cell cycle control, and apoptosis related genes in disease pathogenesis

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Alopecia areata (AA) is a non-scarring inflammatory hair loss disease with an uncertain etio-pathogenesis. It is thought to be a complex polygenic autoimmune disease involving immune deregulation leading to hair loss. The precise susceptibility loci and the molecular basis for the progression of patchy AA to alopecia totalis (AT) and alopecia universalis (AU) are not yet known. In order to better understand the pathogenesis of AA at the molecular level, we investigated the gene expression profiles in skin samples biopsied from AA lesional (n=11) and nonlesional sites (n=11) of AA patients (10 patchy AA and 1 AU) using Affymetrix Hu95A arrays. Hierarchical cluster analysis showed distinct separation of 19 skin samples into disease and normal groups. The lesional AU sample clustered generally with the lesional patchy AA samples but could be distinguished from AA based on a subset of sequences. 87 genes were found to be differentially expressed in AA skin compared to nonlesional skin; 29 were up-regulated and 58 were down-regulated. Validity of microarray data were confirmed by real-time RT-PCR. Functional classification of the 87 differentially expressed genes (DEGs) provides evidence for a T cell mediated immune response (SCYA17, SCYB11, SLA, ICAM1, ITGA2), a possible humoral mechanism (IGHG3) and active antigen processing mechanism (CPVL) in AA. We found modulation in gene expression favoring an arrest of cellular proliferation at various levels (CDK2, FGF18, PPP2R1B, Fe65) with an impairment of DNA repair (BLM, 14-3-3-sigma) and execution of apoptosis (ATM). Further analysis of patients with transient (<1 year duration, n=4) and persistent (>1 year duration, n=7) disease revealed 34 DEGs between the 2 groups (21 up and 13 down-regulated in transient AA). Hierarchical cluster analysis clearly separates transient from persistent AA samples, indicating the existence of gene profiles unique to the initial and later stages of the disease.

0843

Red deer (*Cervus elaphus*) express androgen receptors in their developing antler and antler velvet

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Red deer (*Cervus elaphus*) antlers annually undergo rapid *de novo* development of new bone, skin and hair follicles in the spring. Antler development, and a mane on the neck in the winter season, are secondary sexual characteristics of red deer stags. Although androgens are involved, where they actually act in the antler is unclear. Therefore, we have explored the expression pattern of the androgen receptor in the developing region of antler tips in order to determine potential roles for androgens in antler velvet development. Androgen receptor transcripts were identified using RT-PCR. Positive control tissues, the prostate and testes, showed strong expression of this gene, while heart tissue did not express the androgen receptor gene. Androgen receptors were expressed in all regions of the developing antler tip, which includes the epidermis and dermis, reserve mesenchyme, and developing cartilaginous tissue. Results were confirmed in six different deer for the antler velvet epidermis and dermis; other tissues in at least two deer. In addition, expression was also identified in bald velvet showing only very early hair follicle bud developmental stages. Partial sequence of the androgen receptor in deer shows close similarity with the corresponding region of the bovine (*Bos taurus*) gene. The findings in the developing antler tip suggest that the androgen receptor may play a key role in developing antler cartilaginous tissue. In addition, the androgen receptor may also have a role in the developing epidermis and hair follicles of the antler velvet.

0844

Desmoglein 4 is a novel desmosomal cadherin with an essential role in hair follicle keratinocyte differentiation and is mutated in the lanceolate mouse and localized hypotrichosis in humans

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Desmosomal cadherins are essential cell adhesion molecules present throughout the epidermis. We have identified a novel desmoglein family member, desmoglein 4 (dsg4), using positional cloning in two families with localized autosomal recessive hypotrichosis (LAH). Linkage was established to chromosome 18q21, with a combined multipoint Lod score of 6.29, and comparative genomics identified of the lanceolate mouse (lah/lah) mutation in the same region. In silico cloning revealed several novel human and mouse desmosomal cadherin genes in this region. Among these, Dsg4 was the only novel cadherin gene present in both human and mouse, and thus became a candidate gene for mutations. Sequence analysis revealed an identical 5 kb intragenic deletion in the two LAH families which removes exons 5-8 of DSG4 in frame. Further, we identified mutations in dsg4 in two spontaneous mouse mutations, lah and lah^J. Phenotypic analysis in lah/lah and lah^J/lah^J mutant mice revealed a striking hair shaft defect with the appearance of a lance head within defective hair shafts. Lah hair shafts are devoid of normal cuticle and medullary structures and reveal only a disorganized cortex which keratinizes prematurely, resulting in multiple, periodic lance head deformities. Our data support a crucial role for dsg4 in organizing the differentiation commitments of the keratinocytes in the precortex region of the hair follicle.

0845

Human hair follicle melanocytes express α-MSH and ACTH in a hair growth cycle-dependent manner

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Pro-opiomelanocortin (POMC) is cleaved by pro-hormone convertases (PC)1 and 2 to yield ACTH and a-MSH respectively. These peptides are produced in skin where they participate in important cutaneous functions including melanogenesis. While the involvement of POMC peptides in the regulation of murine hair pigmentation has been well documented, little is known about the role of a-MSH and ACTH in human hair pigmentation/hair growth cycling. This study was designed to assess expression of a-MSH, ACTH and PC1, PC2 and 7B2 in human hair follicle melanocytes (HFM) during the hair growth cycle. Using a double immunofluorescence labelling technique, the expression of a-MSH, ACTH and the POMC processing machinery was strikingly absent in melanogenically-active melanocytes in anagen hair bulbs. However, expression of all markers was marked in a sub-population of poorly differentiated gp100-positive melanocytes in the most proximal and peripheral hair bulb matrix and outer root sheath (ORS) melanocytes. Expression of these markers was also prominent in the follicular papilla, and in the hair bulb matrix and ORS epithelium. The residual bulbs of regressing catagen hair follicles continued to contain a sub-population of gp100-positive melanocytes that expressed a-MSH, ACTH and POMC processing machinery. In telogen, marker expression was evident in gp100-positive cells located primarily in peripheral regions of the epithelial sac of telogen follicles. This study shows significant heterogeneity of POMC expression within the follicular melanocyte pool. Moreover, this expression is coupled to the hair growth cycle, suggesting relevance in follicular melanocyte biology, including melanocyte survival during the apoptosis-driven catagen. Finally, that POMC peptides are prominently expressed in the follicular papilla, but absent in nearby melanogenic bulbar melanocytes, suggests that this hair follicle component may be a local source of these peptides for adjacent pigmenting follicular melanocytes.

0846

A natural canine homologue of alopecia areata in humans

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Alopecia areata (AA) is thought to be an autoimmune disease directed preferentially against hair follicles (HF). Recently, two rodent models of AA were described. Despite several case reports of canine AA, it is not known whether dogs with AA could be useful as an outbred homologue of this disease in humans. The aim of this study was to evaluate 25 dogs with AA and to compare these data with those found in human AA. Biopsies and sera were assessed by histopathology, direct immuno- fluorescence, lymphocyte immunohistochemistry, indirect immuno- fluorescence and immunoblotting analysis of circulating serum IgG, selective immunoprecipitation of HF proteins, and passive transfer of purified canine IgG into naive mice. Clinical signs including alopecia, skin hyperpigmentation and leukotrichia usually developed during adulthood and were first seen on the face. Spontaneous remission occurred in 65% of dogs and regrowing hair shafts were often unpigmented. Histologic examination of skin biopsy specimens revealed mononuclear cell infiltrates affecting almost exclusively anagen HF. Direct immunofluorescence analysis detected HF-specific IgG in 73% of dogs, while indirect immunofluorescence revealed anti-HF IgG to HF inner and outer root sheath, matrix and pre-cortex. Immunoblotting analysis revealed IgG reactivity to 45-60 kDa proteins and a 200-220 kDa doublet. The later was identified as trichohyalin by selective immunoprecipitation. Purified HF-reactive IgG from AA-affected and normal dogs was injected intradermally to the anagen skin of naive mice where it was associated with the local retention of HFs in an extended telogen phase in AA treated skin compared to that seen in controls. In summary, these findings are very similar to those reported for human AA patients and so supports the use of dogs with AA as a useful homologue for the study of the pathogenesis of this common autoimmune disease of humans.

0847

Effects of *Eda* on hair follicle morphogenesis

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In humans and mice, mutations in *Eda* (ectodysplasin-A) cause the abnormal morphogenesis of the hair, teeth, and sweat glands. The Eda protein is a member of the tumor necrosis factor family and known to function in a well-conserved signaling pathway. To investigate the role of Eda in the formation of skin appendages, we generated transgenic mice that express murine *Eda* from the promoter for involucrin (Ivl), a marker of terminal differentiation in the epidermis and hair follicles. In three separate lines of transgenic mice, the *Ivl-Eda* construct caused abnormalities in hair follicle morphogenesis. At the macroscopic level, the trangenic animals diplayed a rough coat, as the hair shafts were disoriented with respect to the body. Within the coat, zig-zag hair was missing entirely, and two new types of hair appeared, derived presumably from abnormal zig-zag follicles. At the histological level, a sub-population of hair follicles underwent aberrant development, leading to anomalous follicle structures. Consistent with the *Ivl* expression pattern, the exogenous Eda was induced at embryonic stage E16.5 and detected in the epidermis and hair follicles during the period of zig-zag hair formation. In all, the results suggest that Eda affects the morphogenetic programs of specific types of hair.

0848

The hedgehog target gene BEG4 potentiates Gli transcriptional responses

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Hedgehog (hh) signaling has diverse, context-dependent functions while controlling the proliferation, differentiation and movement of epithelial cells during development. Additionally, unregulated hh signaling has been shown to be an important contributor to carcinogenesis. The transcriptional output of the hh pathway is largely governed by the Gli family of transcription factors. However, how Gli target genes are selected in different contexts remains largely unknown. We have previously identified a novel hh target gene, BEG4, that is expressed in both human and mouse transgenic basal cell carcinomas. To begin to characterize BEG4s role(s) in skin carcinogenesis we expressed BEG4 in reconstituted human skin grafts in conjunction with transcriptional activators of hh signaling. Skin grafts ectopically expressing Sonic hedgehog demonstrate dramatic increases in keratinocyte proliferation and invasion that are not simulated by grafts expressing Gli1, BEG4 or EGFP alone. However, grafts expressing both hh target genes Gli1 and BEG4 recapitulate the Sonic hedgehog phenotype and demonstrate both robust keratinocyte proliferation and invasion into the dermis. To understand the mechanism of BEG4 action we searched for relevant candidate hh target genes and find that Gli2 regulates the activity of the mouse keratin 17 promoter, a gene highly expressed in hh responsive tissues in skin. Keratin 17 promoter analysis shows that BEG4 dramatically enhances Gli2-dependent transcriptional activity but has no effect alone. Deletion analysis of BEG4 demonstrates that the amino terminus, but not the WH2 domain, of BEG4 is required to potentiate Gli2 transcription. This data is consistent with a BEG4-dependent mechanism for the control of hh target gene expression and indicates that BEG4 may play a key role(s) during organ growth and tumorigenesis.

0849

17β-estradiol inhibits the production of RANTES in human keratinocytes

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A chemokine, regulated upon activation, normal T cell expressed and secreted (RANTES) attracts T helper-1 cells and macrophages. The production of RANTES is enhanced in keratinocytes of psoriatic skin lesions, which may contribute to the inflammatory infiltrate. It is known that estrogen regulates the natural course of psoriasis. We examined the in vitro effects of 17β-estradiol on RANTES production by human keratinocytes. 17β-estradiol inhibited TNF-α or IL-1β-induced RANTES secretion, mRNA expression, and promoter activity in keratinocytes, and these effects of 17β-estradiol were counteracted by estrogen receptor (ER) antagonist ICI 182,780. Two NF-κB elements on RANTES promoter were required for TNF-α or IL-1β-induced transcription and involved in the inhibition by 17β-estradiol. 17β-estradiol inhibited NF-κB transcriptional activity while it did not inhibit DNA binding of NF-κB or phosphorylation or degradation of IκBα in TNF-α or IL-1β-stimulated keratinocytes. 17β-estradiol-induced inhibition of NF-κB transcriptional activity and RANTES promoter activity was rescued by overexpression of a coactivator CREB-binding protein (CBP) or NF-κB p65 but not by steroid receptor coactivator-1 or NF-κB p50. The overexpression of CBP rescued 17β-estradiol-induced inhibition of transcription mediated by a chimeric protein, GAL4-p65$^{286\text{-}551}$, which contained GAL4 DNA binding domain fused to C-terminal transactivating domain of p65 (amino acids 286-551). The transfection of ERα or ERβ into ER-negative SKBR3 cells resulted in 17β-estradiol-mediated inhibition of transcription via GAL4-p65$^{286\text{-}551}$. These results suggest that 17β-estradiol-bound ER may inhibit NF-κB-dependent transcription of RANTES gene by competing with p65 for limiting amounts of CBP.

0850

CD70 is selectively expressed on Th1, but not Th2, cells and required for induction of Th1 dominant-contact hypersensitiveity

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The interaction between the Traf-linked TNF receptor family member CD27 and its ligand CD70 provides a costimulatory signal for T cell activation. Here, we investigated the expression of CD70 on Th1 and Th2 cells, and its contribution to T cell immunity. A low level of CD70 was detectable on resting Th1, but not resting Th2, cells derived from ovalbumin-specific T cell receptor-a/b transgenic mice. After stimulation with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs), the expression of CD70 increased markedly on Th1 cells but remained undetectable on Th2 cells. Similarly, while the CD70 expression was not observed on activated human Th2 clone (D10-G4), CD70 was induced on activated human Th1 clones (28-4, HK-12). CD70 was not detectable on naive T cells, but induced faintly after stimulation. Unlike CD70, similar levels of CD27 were expressed on naive T, Th1, and Th2 cells. Interestingly, CD27 signaling triggered by CD70-transfected P815 cells provided a potent costimulatory activity for naive T cell proliferation and IFN-g production, but exhibited very low costimulatory activity for Th1 and Th2 cell proliferation and cytokine production. We next administered anti-CD70 mAb to BALB/C mice sensitized with dinitrofluorobenzene (DNFB) or fluorescein isothiocyanate (FITC), which has been reported to elicit Th1- or Th2-dominant contact hypersensitivity (CHS), respectively. Administration of anti-CD70 mAb at induction phase significantly decreased the frequency of hapten-specific IFN-g producing cells in regional lymph nodes from mice treated with DNFB, but did not affect the frequency of hapten-specific IL-4 producing cells in regional lymph nodes from mice treated with FITC. These results indicated that CD70 is useful as a marker distinguishing Th1 from Th2 cells, and more importantly, anti-CD70 mAb abrogated the generation of Th1, but not Th2 cells, substantiating a critical role of CD27/CD70 interaction in Th1 development in vivo.

0851

A novel dissociated 1α,25 dihydroxyvitamin D3 analog with immunosuppressive activity in T cell-mediated skin inflammation

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1α,25-Dihydroxyvitamin D3 (Calcitriol), the biologically active form of vitamin D3, is a potent immunomodulatory molecule. However, its clinical use as an immunosuppressant is limited due to its strong effects on calcium homeostasis and the risk of associated side effects. Here, we present a representative of a novel class of vitamin D analogs which exhibits potent immunosuppressive activity in a murine model of contact hypersensitivity (CHS) when applied systemically and is efficacious also at non-hypercalcemic dosages. In vitro analyis revealed a binding affinity of ZK 191784 to the vitamin D receptor comparable to Calcitriol. This compound inhibits dose-dependently lymphocyte proliferation and secretion of TNFα and IL-12 in monocytes but with reduced potency and efficacy than Calcitriol. Treatment of human monocytes with this analog significantly reduces expression of MHC class II, B7.1, and ICAM-1 equipotent to Calcitriol. In vivo, as analyzed in mice the compound potently inhibits the CHS when applied systemically. ZK 191784 has a clear therapeutic advantage over Calcitriol by inducing immunosuppressive effects also at doses that do not cause hypercalcemia. ZK 191784 represents the first representative of a novel class of Vitamin D analogs which might have therapeutic potential in T cell mediated dermatoses.

0852

T cell receptor gene usage in desmoglein-3-specific T lymphocytes from patients with pemphigus vulgaris

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Pemphigus vulgaris is an autoimmune disease mediated by autoantibodies against the target antigen, desmoglein-3. From our previous observations, it is clear that both humoral and cellular autoimmunity play essential roles in the development of pemphigus vulgaris. Recently, we identified that T cells from PV patients proliferate in response to three antigenic fragments on the ectodomain of desmoglein-3. These desmoglein-3-specific T cells are α/β T cells with a CD4 memory T cell phenotype, secreting a Th2-like cytokine profile, and responding to the autoantigen in a restriction to HLA-DRB1*0402 or 1401 alleles. Other characteristics of these autoimmune T cells, such as the usage of T cell receptors, however, have not yet been investigated. The purpose of this study is to determine whether desmoglein-3-specific T cells derived from pemphigus vulgaris patients preferentially utilize certain T cell receptor genes. Using an established reverse transcription polymerase chain reaction method, we found that T cell clones responding to Dsg3 (AA 145-192) preferentially utilize the T cell receptor Vα22Vβ13 genes, while most clones responding to Dsg3 (AA 240-303) and Dsg3 (AA 570-614) utilize Vβ7 and Vβ17 genes, respectively. We believe that this information will further our understanding of the properties of autoimmune T cells in patients with pemphigus vulgaris. Future vigorous investigation, however, is required to analyze the possibility of designing a therapeutic reagent specifically targeting these T cells.

0853

Suppression of contact hypersensitivity reactions by induction of CD4+/CD25+ regulatory T cells after targeting of antigens to immature dendritic cells by anti-DEC-205 antibodies *in vivo*

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Dendritic cells (DC) express a distinct receptor for adsorptive endocytosis, DEC-205. To target antigens to DC in vivo, we coupled the modelantigen ovalbumin (OVA) to anti-DEC-205 mAb (αDEC). Injection of these conjugates into mice lead to efficient uptake of OVA by lymphnode DC and induced proliferation of OVA-specific T cells. However, this expansion is shortlived, and OVA-specific T cells isolated 8 days after injection of αDEC-OVA conjugates failed to proliferate upon restimulation with OVA pulsed DC in vitro. Analysis of those anergic T cells revealed sustained expression of CD25 and CTLA-4 and coculture with conventional CD4+ T cells in MLR assays induced suppression of proliferation and IL-2 production, both of which are functional hallmarks of regulatory T cells. In contrast, these effects were abolished when a DC-maturation stimulus such as a αCD40 antibody was simultaneously injected with αDEC-OVA, suggesting that only non-activated DC induce regulatory T cells in vivo. To further exploit this system for a disease model we injected mice with αDEC coupled to the contact allergen TNCB and we show, that the CD8+ T cell mediated ear swelling reactions were markedly reduced after injection of the αDEC-TNCB conjugates. Similarly, in a CD4+ T cell-driven OVA-specific DTH reaction a reduction of the footpad swelling was obtained after application of αDEC-OVA conjugates. Thus we conclude that targeting of antigens to immature DC via αDEC mAb, leads to induction of regulatory T cells in vivo. These studies provide the basis for a novel strategy to induce regulatory T cells for therapeutic purposes in different autoimmune diseases in vivo.

0854

Antigen-specific suppression of T cell activation in allergy and autoimmune disease models by dendritic cells genetically modified to secrete a T cell receptor mimic peptide

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A T cell receptor mimic peptide (TCRpep) consists of a 8-amino acid peptide, homologous to the transmembrane region of the T cell receptor (TCR) alpha chain. This peptide blocks T cell proliferation by preventing the assembly of an intact TCR. After transfection of Dendritic cells (DC) with the DNA sequence encoding for the peptide, the DCs secreted the TCRpep, causing a reduced antigen-specific T cell proliferation in an OVA specific, transgenic mouse model. To test the effects in vivo, we injected mice with TCRpep transduced DC that had been pulsed with the hapten TNBS. After 6 days mice were challenged with TNCB and ear swelling reaction was determined 24h later. In this CD8-driven CHS model ear swelling reaction was markedly reduced in mice injected with TCRpep transduced DCs as compared to controls. Next we tested the effect of TCRpep tranfected DCs in an experimental autoimune encephalitis (EAE) model, a CD4 dependent murine model for multiple sklerosis. For this, TG4 transgenic mice were injected with TCRpep transduced DC or control DC. After 3 successive injections of DC, the EAE was induced by s.c. injection of the myelinbasic protein emulsified in CFA. Mice in control groups showed significant symptoms of EAE, and 100% died within 3 weeks after induction. In contrast, mice that received TCRpep secreting DC had significantly less severe EAE symptoms and 70% of the animals survived. In a therapeutic approach, EAE was induced first followed by injection of TCRpep secreting DCs. Here EAE was symptoms were suppressed by TCRpep transduced DCs and survival of the mice was significantly improved as compared to controls. This effect was antigen-specific since transfected DC that did not express the respective EAE antigen failed to convey protection. Thus these data show, that DC expressing the TCRpep are able to prevent T cell activation in vivo and might be a useful tool to induce antigen specific immune suppression.

0855

Impact of anatomical location on the requirement of immunoglobulins and B cells for the elicitation of contact hypersensitivity

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Recent studies have uncovered an indispensable function for immunoglobulins (Ig) and B cells in initiating the elicitation of contact hypersensitivity (CH) in mice; however, in these studies CH is elicited by skin painting with antigen (Ag) on one anatomical location, usually the ear. It remains unknown,therefore, whether distinct anatomical locations can respond with different types of immune responses, particularly after chronic Ag exposure. The objective of this study was to investigate the impact of acute or chronic Ag exposure to different anatomical locations on the development of CH. We found that repeated elicitation of CH in the ear of Balb/c and C57BL/6 mice resulted in a shift from a typical DTH to an early-type response with the immediate-type reaction (ITR) followed by late phase reaction (LPR); and the shift was associated with a dramatic increase in serum IgE and mast cell number, indicating IgE-dependent activation of mast cells in the ear. In contrast, elicitation of CH in the footpad led to totally different consequences: ITR followed by LPR was already evident in the acute phase of CH, at the time when serum IgE did not increase; and after chronic Ag exposure there was no further increase in ITR despite an increase in serum IgE, suggesting IgE-independent activation of mast cells in the footpad. To examine further whether Ig and B cells can be critically involved in the elicitation of CH on diverse locations, B cell-deficient C57BL/6 (μ MT-/-) mice were elicited using the same protocol. Surprisingly, even in the acute phase, μ MT-/-, only when elicited in the footpad, developed accelerated CH responses with an early-type response and a major DTH reaction after 24h; and after chronic Ag exposure a dramatic increase in the early-type response was detected, indicating that neither Ig nor B cells are needed for the elicitation of CH in the footpad. Ig and B cells indispensable for the elicitation of CH in one anatomical location may, on the contrary, serve to inhibit the development of CH in other locations.

0856

Polymorphic expression of CD158k/P140/KIR3DL2 in Sezary patients

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Sezary syndrome (SS) is a leukemic form of epidermotropic cutaneous T-cell lymphoma (CTCL). No specific cell menbrane receptor has been described until now on Sezary cells. Recently, we reported that circulating and cutaneous sezary cells express CD158k/P140/KIR3DL2. This menbrane receptor is a member of the killer cell Ig-like receptors that inhibit NK-mediated lysis after interaction with HLA-A. In normal individual KIR3DL2 is only detected on minor NK, and CD3+CD8+ subsets. Until now, nine KIR3DL2 alleles have been defined. We reported the restricted expression of KIR3DL2 008 allele from 2/2 CTCL lines derived from Sezary patients, suggesting that expression of this allelic form could be associated to CTCL malignancies. The role of KIR3DL2 on CTCL cells is still unknown. It could be possible that KIR3DL2 polymorphism might influence the recognition of HLA-I alleles. In order to determine whether Sezary cells express a specific KIR3DL2 allelic form, we have determined the repertoire of KIR3DL2 expression in 14 patients presenting SS. CDNA was PCR-amplified througg 40 cycles, with two different conditions depending on the KIR3DL2 allele specific primers.The amplification products were analysed on a 1% agarose gel. 10/14 patients expressed two different alleles including 009/007 (1 patient), 002/005 (1 patient), 007/006 (1 patient), 002/007 (3 patients), 003/007 (3 patients), 005/007 (1 patient). Whereas 4/14 patients were homozygote including 009 (2 patients), 002 (1 patient), 001 (1 patient). None of the patients was homozygote for KIR3DL2 008 found expressed in CTCL line. These results demonstrate that there is no preferential allelic expression of KIR3DL2 associated with Sezary syndrome.

0857

Engagement of the high affinity IgE receptor on human monocytes induces the secretion of soluble factors that inhibit IgE production and augment IgG4 synthesis

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Although exposure to inhaled allergens often induces naturally developed or immunotherapy-induced tolerance in patients with atopy, the precise mechanism is unclear. In this presentation, we studied the role of the high affinity IgE receptor (FcεRI) expressed on antigen presenting cells in the production of IgE and IgG4 by B cells. Peripheral human blood monocytes were obtained from patients with atopic dermatitis and non-atopic healthy individuals and enriched by density centrifugation. Culture supernatant of monocytes (Sup-Fc) was harvested after 24 hr from engagement of FcεRI and the effect of Sup-Fc on IgE and IgG4 production by peripheral blood mononuclear cells (PBMC) and purified B cells was examined. Sup-Fc downregulated IL-4 and/or sCD40L-induced IgE production and enhanced IgG4, IgG1-3, and IgA production by PBMC without affecting their proliferation. Sup-Fc also suppressed germline and mature Cε transcripts while it augmented Cγ4 transcripts in PBMC. However, Sup-Fc upregulated IL-4 and/or sCD40L-induced IgE and IgG4 production by purified B cells. The differential effects of Sup-Fc on the IgE and IgG4 production were blocked with neutralization antibodies against IL-10 and IL-8. These results suggest that aggregation of FcεRI on monocytes induce the secretion of soluble factors that suppresses IgE class switching and may explain the role of antigen presenting cells in FcεRI-mediated tolerance against inhaled allergens.

0858

Transfection of dendritic cells with antigen-encoding RNA generates a cellular vaccine that induces protective CD8 T cell-dependent antiviral immunity

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Dendritic cells (DC) are obvious targets for antigen-specific immune intervention. Here we attempted to introduce a CD8 T cell-dependent epitope into DC for presentation on MHC class I and induction of antiviral immunity. Bone marrow-derived DC were subjected to electroporation with RNA transcribed in vitro from a plasmid encoding lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp) or green fluorescent protein (GFP) under the control of a T7 promotor. Cells were then incubated with LPS to induce maturation of DC. LPS-treated GFP+CD11c+ cells upregulated MHC class II, CD40, CD80 and CD86 as well as GFP-CD11c- cells or untreated cells from control cultures, indicating preserved maturation of transfected DC. DC expressing LCMV gp but not DC expressing GFP or untreated DC induced cell number dependent in vitro proliferation of CD8 T cells expressing a transgenic receptor recognizing the 33-41 sequence of LCMV gp in association with H-2K/D^{b}, indicating correct synthesis, processing and presentation of LCMV gp by transfected DC. Transfected DC were then injected intravenously twice in weekly intervals into naive C57/BL6 mice and challenged one week after the last DC injection with 200 PFU of LCMV intravenously. Five days later the viral titer in the spleen from mice that received LCMVgp expressing DC but not GFP-expressing DC or untreated control DC was 5 to 7 logs lower than in nonvaccinated but virus challenged mice. These results indicate that targeting DC with antigen-encoding RNA can induce antigen-specific CD8 T cell responses and more importantly protective antiviral immunity in vivo. Targeting DC with antigen-encoding RNA may find wider application for immune intervention in disorders such as autoimmunity and cancer in addition to vaccination for viral diseases.

0859

Imbalance of CD123+ (lymphoid) and CD11c+ (myeloid) dendritic cells is critical for Th2 polarization in atopic dermatitis

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Dendritic cell (DC) is a professional antigen-presenting cell in the immune system, orchestrating the decision-making process in the differentiation of pre-Th cells into Th1 and Th2 cells. In lymph nodes and circulation in human,two DC subsets, DC1 and DC2, are identified based on origin, phenotype and function. DC2 has a function to drive Th2 development and provides the milieu favoring Th2-biased immune reactions. Recent reports have provided evidence that the number and balance of blood DCs reflect the host's immune status. Atopic dermatitis (AD) has characteristic features of marked Th2 polarization such as Th2 cytokine expression in the lesional skin and enhanced IgE production and eosinophilia in the blood. We found that lower ratio of blood DC1/DC2 subsets in AD as compared with psoriasis and normal subjects. The ratio was also closely associated with the extent of Th2 polarization, as exemplified by the serum levels of total and specific IgE,Th1/Th2 cytokine production of peripheral blood cells and the disease severity estimated by SCORAD. Therefore, the degree of DC imbalance in circulation is suggested to be one of the critical factors that determine atopic skin inflammation.

0860

Epidermal involvement and mechanism of ulceration in the pathogenesis of cutaneous leishmaniasis

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Cutaneous leishmaniasis (CL) is a vector-born disease caused by flagellated protozoa of the genus *Leishmania*. We use a murine model and histoimmunofluorescent techniques to study the kinetics of ulceration and the level of epidermal involvement during CL caused by *L.major*. We studied the epidermis associated with cellular infiltrate in susceptible BALB/c mice at 7 weeks after intradermal infection with 500 parasites. Following injection and a 4 week incubation period, a red-furuncle-like nodule appears at the site of inoculation. The nodule ulcerates two weeks later and then enlarges. The biopsies revealed mixed infiltrates characterized by a predominance of lymphocytic cells associated with polymorphonuclear leukocytes. The microanatomic localization of the infiltrate was diffuse in the dermis. Ulcer development was associated with activated $CD11c^{+}CD8\alpha^{-}$ Langerhans cell (LC) present in the place of future disruption. Each LC was surrounded by MHC class II^{+} keratinocytes, in such a manner "a nest of activation" was formed. Keratinocytes in the place of the future ulcer gradually increased MHC class II expression. At this time the exocytosis was presented, that is infiltration of mononuclear cells into the epidermis, accompanied by spongiosis. The dominant type of the exocytotic cells were T lymphocytes (73.2%), the most part of which was $CD4^{+}$ T cell producing IL-4 and TNFα. Also activated $F4/80^{+}$ $iNOS^{+}$ macrophages (18.3%) and $CD8^{+}$ pan-NK^{+} cells (8.5%) were present in the epidermis, and NK cells, macrophages and lymphocytes were concentrated under the upper layer of papillary dermis at the same time. Furthermore, we observed a high level of IFNγ, TNFα and IL-4 production directly under the epidermis. Immediately following these observations, ulceration ensued. We hypothesize that up-regulation of MHC class II in this population of keratinocytes goes parallely with down-regulation of MHC class I that is a signal of NK cells attack, lysis and form a defect of tissue. Thus, this phenomenon emphasizes the importance of the epidermis in the immunoregulation of the disease.

0861

Human mast cell development in mice after xenotransplantation of cord blood-derived hematopoietic stem cells

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Recently stem cell factor (SCF)-dependent in vitro-derived human mast cell (MC) culture system has been established, which provides us wonderful opportunities for the investigation of human MC functions. However to study the development mechanism and functional roles of human MC in our body, the establishment of in vivo model is desired. The transplantation of primitive human cells into immune-deficient mice is the well-established in vivo system for the investigation of human hematopoietic stem cell (HSC) function. Although MCs are the progeny of HSCs, human MC development after the transplantation of human HSCs into mice has never been reported. We newly developed NOD/SCID/γ_c^{null} (NOG) mice, double homozygous for the severe combined immunodeficiency and IL-2 receptor common γ-chain defect mutation, and reported that NOG mice were the excellent recipient animals for human cell engraftment. Fifty thousand of enriched CD34+ cells from cord blood were injected into sublethally irradiated NOG mice intravenously through the tail vein. Twelve weeks after the transplantation, approximately 42% of peripheral white blood cells represented human CD45+ in NOG mice. Compared with other reconstituted blood cells, however, the appearance of human MCs in NOG mice was delayed; only 12 weeks after the xenotransplantation, a very small number of human CD45+ MCs were recognized focally in the upper dermis, then they gradually increased in a number supported by murine SCF. By immunohistochemical staining, human MCs in the mouse dermis were chymase positive cells. Human MCs were also developed in mouse lung and gastric tract, where MCs containing both tryptase and chymase distributed in submucosa and around big airways, whereas MCs containing tryptase but lacking chymase located in mucosa, just as in our human body. Xenotransplantation of human HSCs into NOG mice shows the reconstitution of blood cells including MCs, which is a potentially nice tool for the investigation of human MC development and functions in vivo.

0862

Drug specific cytotoxic CD8 T-lymphocytes as effector cells in toxic epidermal necrolysis

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In a patient suffering from sulfamethoxazole (SMX) related TEN, we recently reported the presence in the blister fluid of diseased skin of CD8 cytotoxic T-lymphocytes (CTL). Like classical CTL they killed autologous lymphocytes in a drug specific, class I restricted and perforin/granzyme mediated pathway. We report here additional findings in a total of 6 patients. Blister fluid were collected on admission (2 to 6 days after onset of disease) in 6 patients with TEN, 5 related to " highly suspected " drugs (SMX 3, carbamazepine 2) and 1 with recent exposure to a usually non-suspected drug (tetrazepam). In all 6 cases the cells present in blister fluids were mainly lymphocytes (40 to 75%, which were mostly CD8+ (53% to 99%), CLA+ (54% to 83%), and often CD56+(21% to 72%) and HLA-DR+ (20% to 85%). In 4/6 cases (3 SMX, 1 carbamazepine) blister fluid lymphocytes exhibited a drug specific cytotoxicity on autologous EBV transformed lymphocytes. This was found in the presence of therapeutic concentrations of the " parent " drug. Cross reactivity was observed between SMX and some other sulfonamides (sulfamethizole, sulfadiazine and sulfapyridine), but not between SMX and sulfasalazine or other sulfonamide related drugs (chlorpropamide, tolbutamide, glyburide, hydrochlorothiazide). In addition in one case keratinocytes were obtained from hair follicles and used as targets. After activation with interferon gamma autologous keratinocytes were lysed by blister fluid lymphocytes in the presence of SMX. Taken together these results suggest that CTLs play a major role in the widespread death of keratinocytes, which characterize TEN. In many cases these CTLs are drug specific. In other cases it remains to determine whether another drug, a metabolite or another antigen elicited the reaction.

0863

Serum soluble P-selectin glycoprotein ligand-1 was up-regulated in patients with atopic dermatitis

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P-selectin is expressed on endothelial cells. P-selectin ligand glycoprotein ligand-1 (PSGL-1), which was first identified on myeloid cells, is a homodimer of two about 120 Kd subunits. PSGL-1 plays a crucial role on the early inflammation stage, especially leukocyte rolling. Moreover, topical soluble PSGL-1 (sPSGL-1) injection in mice with allergic conjunctivitis reduce the inflammation. We studied the serum concentration of sPSGL-1 on the patients with atopic dermatitis and psoriasis vulgaris. In the patients with atopic dermatitis, sPSGL-1 was upregulated in comparison with healthy control ($p<0.05$). Moreover, there was a high correlation between the sPSGL-1 and disease activity, SCORAD (SCORing Atopic Dermatitis). However, in the patients with psoriasis vulgaris, sPSGL-1 showed neither significant up nor down regulation with healthy control. We thought that sPSGL-1 might be a the disease activity marker with atopic dermatitis.

0864

Inhibition of Th2 cytokine production by regulatory CD4+CD25+ T cells in allergic/atopic individuals

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CD4+CD25+ regulatory T cells from human peripheral blood have been demonstrated to inhibit allogeneic proliferation and cytokine production of preactivated CD4+CD25- responder T cells. The aim of this study was to analyze in an allergen-specific setting whether such regulatory CD4+CD25+ T cells also exist and function normally in atopic individuals, especially concerning the inhibition of Th2 cytokines. For this purpose, CD4+CD25- or CD4+CD25+ T cells from grass or birch pollen allergic donors were stimulated in the presence of autologous mature monocyte-derived allergen-pulsed dendritic cells and the preactivated CD4+CD25+ T cells were added to CD4+CD25- T cells during restimulation. CD4+CD25+ T cells from the majority of the patients investigated proliferated poorly, produced less cytokines and inhibited the proliferation as well as the Th1 (IFN-gamma) and Th2 (IL-4, IL-5) cytokine production of CD4+CD25- T cells, but not IL-10 production. The suppression of CD4+CD25- T cells by CD4+CD25+ T cells was mainly antigen-unspecific and not reversible by anti-IL-10, anti-TGF-beta or anti-CTLA-4 mAb, but by IL-2. In some atopic patients, preactivated CD4+CD25+ T cells reproducibly showed strong proliferative responses, produced higher amounts of IL-4 and IL-10 than CD4+CD25- T cells and suppressed only the IFN-gamma production of CD4+CD25- T cells. These data indicate that regulatory CD4+CD25+ T cells are present and functional in most atopic patients with allergic rhinitis and are able to inhibit Th1 as well as Th2 cytokine production.

0865

PGE2 produced by DCs acts T cells to enhance differentiation into Th1 and Tc1 cells in contact hypersensitivity

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Prostaglandin (PG) E2 mediates its physiological role through EP1, EP2, EP3, and EP4. In immune system, only EP2 and EP4 are known to suppress T cell function. Here we found that mRNA of EP1 was also abundantly expressed on CD4+, CD8+, and CD11c+ cells using RT-PCR. However, the role of EP1 in immune system remains completely unknown. To evaluate this issue, we used newly generated EP1knockout(KO) mice and EP1 selective compounds. In contact hypersensitivity (CHS) model, EP1KO mice showed significant suppression in both ear swelling and total cell counts of cervical lymphocytes after challenge with DNFB compared to control C57BL/6 mice. The number of IFN-γ producing CD4+ and CD8+ cells from regional lymph nodes after challenge were also decreased in EP1KO mice. Moreover, when draining lymph node cells after sensitization were stimulated with DNBS in vitro, the level of IFN-γ in culture medium was significantly decreased in EP1KO mice. Suppression of CHS response was reproduced pharmacologically when the EP1 antagonist was administered during the sensitization phase to control mice. Consistently, mRNA of PGE synthase was elevated in regional lymph nodes 1-5 days after sensitization and the level of PGE2 were abundant in the culture medium of DCs. When we evaluated the function of DCs using FITC-induced Langerhans cell migration and mixed lymphocyte reaction assay, the EP1 selective compounds did not modulate the migration and T cell stimulation activity of DCs. Therefore, we then assessed the effects of EP1 on T cell function. Naive CD4+ and CD8+ cells were stimulated with anti-CD3 under Th1 (IL-2, IL-12 and anti-IL-4 Ab) or Th2 (IL-2, IL-4 and anti-IL-12 Ab) driving condition, the EP1 selective agonist increased the number of IFN-γ cytokine but decreased the number of IL-4 cytokine producing CD4+ or CD8+ cells. These results suggest that PGE2 produced by DCs in the regional lymph nodes during sensitization phase of CHS acts T cells in situ to enhance differentiation into Th1 and Tc1 cells.

0866

Prostaglandin E2-EP4 signaling is critically involved in migration and maturation of Langerhans cells in the initiation phase of contact hypersensitivity

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The antigen-specific immune response in the skin is initiated by antigen ingestion by Langerhans cells (LCs) in the epidermis and their migration to draining lymph nodes. While prostaglandin (PG) E2 is produced abundantly during antigen exposure and accompanying inflammation in the skin, the role of PGE2 in the initiation process remains unknown. We examined this issue by using mice deficient in four subtypes of PGE receptor, EP1 to 4, individually, and EP subtype-selective drugs. We found that, while LCs expressed all EP subtypes, the number of LCs that migrated to the regional lymph nodes after FITC application was significantly decreased only in EP4KO mice. The decreased LC migration was mimicked in C57BL/6 mice treated with an EP4-selective antagonist. Consistently, an EP4 agonist increased the number of LCs that migrated to the culture medium from the mouse skin explants, and the number of migrated LCs towards CCL19, as a CCR7 ligand, through a trans-well. Moreover, an EP4 agonist significantly increased expression of co-stimulatory molecule of LCs in the organ culture, and acted on LCs in mixed lymphocyte reaction assay to enhance allogeneic T cell proliferation and activation. On the other hand, the morphology and density of LCs in epidermal sheets in the steady state, and the endocytotic activity using FITC-dextran are comparable between EP4WT and EP4KO mice. Finally, in vivo in the contact hypersensitivity model, the delayed-type hypersensitivity response was significantly impaired in EP4KO mice compared to EP4WT mice. This impaired response was reproduced in C57BL/6 mice treated with the EP4 antagonist during sensitization period. The antigen-specific T cell proliferation and Th1 cytokine production was also significantly reduced in cells form the regional lymph nodes of EP4KO mice. Taken together, our results suggest that the PGE2-EP4 signaling is critical in migration and maturation of LCs in the initiation phase of CHS.

0867

PKC and MMP inhibitors as potential modulators of Langerhans cell migration following hapten-induced sensitization

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Langerhans cell (LC) migration from the epidermis to draining lymph nodes is a critical first step for the sensitisation as well as for the initiation of contact hypersensitivity. Among the intracellular mechanisms which mediate LC migration from the skin, pro-inflammatory cytokines, protein kinase C (PKC) and Matrix Metallo Proteinases (MMP) activation seem to play a pivotal role. In the present work, we have investigated the efficiency of different PKC inhibitors alone, or in association with an MMP inhibitor in modulating the migration of activated LC. The migratory capacity of freshly isolated LC was assessed using a reconstituted basement membrane assay (Matrigel) mimicking the prerequisite passage through the dermal-epidermal junction on the way to lymph nodes. Suspensions highly enriched in Langerhans cells were treated for 1h with different concentrations of inhibitors of PKC and/or MMP inhibitor before being exposed to a strong sensitizer, DNBS, and subjected to migration using modified Boyden chambers. Our results demonstrate that all the PKC inhibitors (with large or narrow specificity) as well as the MMP inhibitor are per se potent modulators of activated LC migration. Moreover, these two kind of compounds seems to act in an additive way. Indeed, in the presence of both inhibitors, activated LC migration rate is comparable to those of non activated cell. Thus, PKC and MMP inhibitors can act as potentially important modulators of contact hypersensitivity. LC migration is also under the control of pro-inflammatory cytokines such as IL-1b and TNF-a. The cytokine profile of activated LC treated with both type of inhibitors is under investigation.

0868

Pimecrolimus protects mice from developing T cell-induced inflammatory bowel disease without systemic immunosuppression, in contrast to tacrolimus or cyclosporin A

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Oral pimecrolimus is active in patients with psoriasis or atopic dermatitis, inflammatory skin diseases which share many pathogenic features of inflammatory bowel disease (IBD). Using a T cell induced mouse model of severe IBD, we compared the activity of calcineurin inhibitors, pimecrolimus, tacrolimus and cyclosporin A (CyA). SCID mice were reconstituted with $2x10^5$ CD4+CD45RBHi T cells and treated orally once a day for 4 weeks with pimecrolimus (30, 60 or 100mg/kg/d), tacrolimus (3, 10 or 30mg/kg/d) or vehicle. CyA (Sandimmun®) was administered continuously via s.c. mini-pump (15, 30 or 60mg/kg/d). We measured body weight, serum haptoglobin, neutrophil (Ly-6G+ cells) and T (CD3+CD4+) cell numbers by FACS analysis of single cell suspensions from blood, spleen and mesenteric lymph nodes (MLN). Colitis was assessed histologically. All doses of pimecrolimus tested prevented, whilst tacrolimus and CyA accelerated the severe weight loss. Colon sections showed a significant ($p<0.01$) reduction in inflammation in pimecrolimus (60 and 100mg/kg/d)- relative to vehicle-treated mice. A significant reduction in colitis in tacrolimus-treated mice was only achieved consistently with the side-effect inducing dose of 30mg/kg/d. CyA treatment, whilst toxic, was effective ($p<0.01$) at 60 but not at 30mg/kg/d. All 3 inhibitors significantly reduced serum haptoglobin levels and neutrophilia in blood and spleen. Pimecrolimus, at the high dose of 100mg/kg/d, caused no significant reduction in T cell numbers in the blood (-19.6±20.0; n=3 separate studies) or spleen (23.7±13.0) but induced a 61.0%±7.3 reduction in MLNs. In contrast, tacrolimus (10mg/kg/d) reduced T cell numbers in blood, spleen and MLN by 72.3%, 83.1% and 80.2%, respectively, compared to an 85.1%, 68.1% and 63.6% reduction by CyA (30mg/kg/d). In conclusion, these data suggest that pimecrolimus prevents IBD by selectively regulating the intestinal immune system whilst tacrolimus and CyA induce general immunosuppression and severe side effects.

0869

Blockade of skin dendritic cell migration by topical application of MMP- and PKC- inhibitors

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Epidermal langerhans cells (LC) and skin dendritic cells (DCs) are potent antigen presenting cell (APC) in the induction of primary T cell-mediated immune responses in the skin. They capture foreign antigens (Ags) and migrate to regional lymph nodes (LNs) to carry and present this antigen to naive T cell. This mechanism is critical in the initiation of different cutaneous pathology such as Psoriasis, Contact Dermatitis or Contact Hypersensitivity (CHS). Several molecules regulate the migration of skin DC, among which metalloproteinase (MMPs) and proteine kinase C (PKC). The aim of this study was to evaluate the participation of MMPs and PKC in the in vivo migration of murine skin DCs, using topical inhibitors for MMP and PKC. Mice were ear painted with FITC and DC migration to auricular LNs was assessed 24 hrs later by flow cytometry quantification of FITC+, CD86+, MHC class II high positive cells. Topical application of MMP or PKC inhibitors on the skin once a day for 4 consecutive days induced a dose-dependant reduction in the number of FITC+ migrating DC (around 30% of inhibition for the optimal dose), compared to placebo application. When PKC and MMP inhibitors were mixed in the same ointment, the inhibitory effect was more pronounced (86% of inhibition), suggesting a synergistic action. These data indicate that, topical use of inhibitors of PKC plus MMPs, could inhibit skin DC migration, which could have strong therapeutic implications in the treatment of skin inflammatory dermatoses.

0870

LFA878: a statin-derived leukocyte function-associated antigen-1 inhibitor with high potency against rodent allergic contact dermatitis

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Leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) is a member of the β2 integrin family which is expressed on all leukocytes. The interaction of LFA-1 with its most important counter-receptor ICAM-1, mediates cellular interaction in the immune system and extravasation/migration of leukocytes to sites of inflammation. Blocking the LFA-1/ICAM interaction, therefore, is considered to be a therapeutic strategy in inflammatory diseases. Clinically, this approach is followed by the parenteral use of anti-LFA-1 antibodies in patients with psoriasis. An in-house *in vitro* screening revealed the HMG-CoA reductase inhibitor lovastatin as a weak inhibitor of the LFA-1/ICAM-1 interaction. This finding prompted the synthesis of lovastatin- derived molecules including LFA878 which potently inhibit LFA-1 but lack the effect of HMG-CoA reductase. LFA878 selectively inhibits *in vitro* the LFA-1/ICAM-1 interaction with IC50s of 50 ± 2 nM (cell-free binding assay) and 300 ± 150 nM (cell-based adhesion assay). LFA878 up to 100 µM did not affect binding mediated by the closely related β2 Mac-1 (CD11b/CD18) and the β1 integrin VLA-4 (CD49d/CD29). Parenteral administration of anti-LFA-1 antibodies has been shown to inhibit contact hypersensitivity in mice. Therefore, this disease has been used as a model of T-cell mediated skin diseases for profiling LFA878 in rodents. The compound potently inhibited contact hypersensitivity at minimal effective oral doses of 0.1 mg/kg in mice and 0.8 mg/kg in rats, respectively. Since findings in this model are very predictive for the human situation, LFA878 is considered to be a potential drug candidate for oral treatment of allergic contact dermatitis and other T cell dependent skin diseases such as psoriasis.

0871

Pimecrolimus potently inhibits established severe disease in SCID mice with T cell-induced inflammatory bowel disease

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Oral pimecrolimus is therapeutically active in patients with psoriasis or atopic dermatitis, diseases sharing many pathogenic features of inflammatory bowel disease (IBD). Using a mouse model of severe, established T cell induced IBD we show that pimecrolimus possesses potent therapeutic activity. SCID mice were reconstituted with $2x10^5$ CD4+CD45RBHi T cells. Pimecrolimus (100mg/kg/d), or vehicle, was given orally to mice from day 7, 14 or 21 after T cell transfer and then daily until day 30. We measured body weight (BW), serum haptoglobin, neutrophil (Ly-6G+ cells) and T (CD3+CD4+) cell numbers by FACS analysis of cell suspensions from blood, spleen and mesenteric lymph nodes (MLN). Colitis was assessed histologically and myeloperoxidase (MPO) was assayed in colon homogenates as a marker of neutrophil infiltrate. Initiating pimecrolimus treatment on day 21 post T cell transfer rapidly reversed the loss in BW. Starting treatment earlier prevented BW loss. In untreated mice the colon inflammation by day 21 is usually scored as severe. When pimecrolimus treatment was started on day 21, the mean score by day 30 was significantly ($p<0.01$) reduced to moderate, and when starting on day 7, to low scores describing only mild inflammation. The neutrophil infiltrate, measured as MPO activity, in diseased colons of the 21d delayed mice was potently reduced by 68% relative to the vehicle-treated group by day 30. Pimecrolimus reduced both the serum haptoglobin levels (by 86 and 97%, n=2 studies) and nearly totally inhibited the disease-induced neutrophilia in the blood and spleen of severely diseased mice. FACS analysis showed that pimecrolimus was not preventing activated T cells from proliferating as numbers were not reduced in the pimecrolimus- relative to vehicle-treated mice. Pimecrolimus is highly effective in a therapeutic setting, strongly inhibiting some of the downstream systemic inflammatory reactions. The data from this severe chronic model of IBD lead us to expect pimecrolimus to be effective in IBD in man.

0872

Development of a novel strategy to generate mice lacking dendritic cells

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Dendritic cells (DCs) occupy a central location in immune regulation and are responsible for both the development of immune responses and the generation of peripheral tolerance. Dissecting their precise role in immune regulation, however, has been hampered by the absence of a mouse model specifically lacking dendritic cells. We are in the process of developing a murine model with a specific defect of dendritic cells. The first step of the strategy is to generate transgenic (Tg) mice from unmodified human Bacterial Artificial Chromosome (BAC) DNA that contains a putative DC-specific gene and document that Tg expression is indeed specific for DCs using antibodies to the human proteins. BACs containing DC-specific genes will then be modified to express diphtheria toxin and the modified BAC used to make a second round of Tg mice. These Tg mice should lack DCs or DC subsets owing to the expressing of diphtheria toxin from the Tg. We have generated two lines of Tg mice using an unmodified human BAC containing the gene for DC-SIGN and another line containing the gene for Langerin. DC-SIGN is expressed on all immature human DC lineages but is absent from macrophages and Langerhan cells. Langerin is expressed primarily on epidermal Langerhan cells. FACS analysis of bone marrow derived DCs and peripheral lymph nodes from DC-SIGN Tg mice reveals huDC-SIGN expression on a subset of CD11c+ cells. By immunohistochemistry, the huDC-SIGN Tg is expressed on a large number of cells with dendritic morphology in the T cell area of the lymph node and on rare cells in the dermis and colonic adventitia. These cells are primarily of the "myeloid" DC type and are CD11b+, CD4+, and CD8-. Preliminary analysis of the Langerin Tg mice shows strong expression of the human Langerin Tg in Langerhan cells but not in dermal DCs. Thus, human BAC DNAs containing Langerin or DC-SIGN appear able to confer specific Tg expression in particular DC subsets. We have successfully modified the BACs containing Langerin and DC-SIGN to express diphtheria toxin and are in the process of generating new Tg lines with these modified BACs.

0873

Characteristics of antibody responses to a polyvalent melanoma vaccine

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There is increasing interest in constructing cancer vaccines from multiple tumor antigens to improve their effectiveness. Little is known about the characteristics of the antibody responses induced by such vaccines. The following study was conducted to address this question. The study was conducted on 188 pts with resected stage IIb-IV melanoma who were immunized with a polyvalent, shed antigen, melanoma vaccine. 62% had AJCC stage IIb or IIIa, 21% had stage IIIb, and 17% had stage IV melanoma. Immunizations were given intradermally q2-3 wks x 4 and then monthly x 3. IgM and IgG antibodies to antigens in a detergent extract of melanoma cells were measured by western immunoblotting at baseline and following 4 (IgM responses) or 7 (IgG responses) immunizations. Vaccine-induced antibody responses were defined as a new or enhanced response following immunization compared to baseline in the same pt. Fifty two percent of pts had a vaccine-induced anti-melanoma IgM and/or IgG response. IgM and IgG responses were induced in a similar proportion of pts (31% vs 32%). There was no relation between stage of disease and the frequency or class of response. The responses were directed to one or more antigens ranging in size from 35 to 185 kds. There was considerable heterogeneity in the pattern of antigens recognized by individual pts and no antigen was clearly immunodominant. In the majority of responding pts (76%) the responses consisted of an increase in the level of a melanoma antibody that was already present prior to vaccination, rather than the induction of a new antibody response. These results indicate that antibody responses to polyvalent vaccines are complex and directed to a broad range of antigens. Different pts develop responses to different sets of these antigens. Immune responses do not decrease in more advanced disease. The most novel finding was that immunization enhanced pre-existing anti-melanoma antibody responses rather than induce new responses.

0874

Regulation of human β-defensin 3 in human keratinocyte (HaCaT) cell lines

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The large surfaces of the skin are often initial site of contact between microorganism and human. Recent studies introduce us new peptides with antimicrobial activity such as β-defensins and cathelicidins. They are expressed on the epithelia and polymorphonuclear leukocytes, which are first lines of defence from various invasive environments. Futhermore, they are considered very interesting and important endogenous antibiotics. Our previous study has shown that the expression of human defensin(hBD)-2 mRNA, which is potent antibiotic peptide against Gram-negative bacteria(P. aeruginosa), was upregulated with ultraviolet(UV) irradiation, tumor necrosis factor-α(TNF-α) and lipopolysaccharide(LPS) in HaCaT cells. A novel hBD-3, 5-kDa, nonhemolytic antimicrobial peptide, was demonstrated a salt-insensitive broad spectrum of potent antimicrobial activity against many potentially pathogenic microbes in especially, multiresistant S. aureus. We have analyzed the expression patterns of hBD-3 in HaCaT cell lines. This research has done in order to evaluate the expression and regulation of hBD-3 mRNA in human keratinocyte cell lines. HaCaT cell lines were used to all culture experiments. Cultured human keratinocytes were stimulated with UV irradiation or TNF-α or LPS to determine whether hBD-3 mRNA production occurred. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify hBD-3 cDNA from stimulated keratinocytes in a time dependant manner, and densitometry was used to verify the specificity of RT-PCR amplication products. Expression of hBD-3 was upregulated with UV irradiation, TNF-α and LPS in HaCaT cells compared to control. Human keratinocytes are capable to induce hBD-3 mRNA, as well as hBD-2, in response to UV irradiation, TNF-α and LPS suggesting that these cells could play an important role against the bacterial infection and UV light damage in human skin.

0875

Differential expression and function of toll-like receptor in murine Langerhans cells: comparison with splenic dendritic cells

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We purified murine resident epidermal Langerhans cells (LC) using panning method and studied the expression pattern and functions of Toll-like receptors (TLRs) of LC. We found the following: (1) LC expressed TLRs 2, 4, 9, but not 7, the pattern of which suggest LC are the closest to one of the splenic dendritic cell (DC) lineage, CD11c+11b+8αα-4-; (2) TLR4 and its accessory molecules CD14 and Rp105 (CD180) are more highly expressed on splenic DC than on LC; (3) murine LC, similar to human LC, overall produced Th1 cytokines including IL-12p40 and IP-10 on stimulation to each expressed TLR, namely, to TLR2 with Staphylococcus aureus Cowan I (SAC), to TLR4 with lipopolysaccharide (LPS), and to TLR9 with unmetylated CpG; only exception was IL-12p40 unresponsiveness on stimulation with LPS; (4) on the other hand, the production of Th2 cytokine, TARC/CCL17 was decreased on stimulation with these TLR stimulations; (5) the IL-6 production by LPS-stimulated LC was significantly lower than that by LPS-stimulated DC, whereas the TARC production was higher in LC than in DC. The difference in the responsiveness to LPS between epidermal LC and splenic DC might be due to the deviated expressions of TLR4 and its accessory molecules. These results may represent the heterogeneity of DC lineages and functions.

0876

Classification of immunoregulatory stimuli by dendritic cell-based biosensor

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Dendritic cells (DC) are uniquely equipped with many receptors, including Toll-like receptors (TLR) and P2 receptors (P2R), to recognize molecular patterns produced by microorganisms, endogenous "danger" signals, and various soluble and membrane-associated immunoregulatory molecules. Ligation of these receptors triggers rapid and dynamic reprogramming of gene expression and cellular functions, the process known as DC maturation or activation. We reasoned that any agent that turns on particular transcription regulatory pathway(s) by binding to DC-associated receptors must alter DC function in a predictable manner. To test this concept, we created a multi-channel, DC-based biosensor system by transducing XS106 DC line with Luc reporter constructs driven by each of 12 different *cis*-enhancer elements. By measuring Luc activities following exposure to test samples, we have identified a diverse array of DC-stimulating agents, including TLR ligands (LPS and CpG oligonucleotides), other microbial products (muramyl dipeptide, D-mannose, and Protein A), P2 receptor ligands (ATP and ADP), skin irritant chemicals (croton oil and ethyl phenylpropiolate), pharmacological agents (PMA and Forskolin), immuno-modifiers (cytokines), crude biological materials (necrotic keratinocytes), and physical stimulus (phagocytosis of latex beads). The system has further allowed us to classify these agents based on the unique activation profiles of transcription regulatory pathways (e.g., NFkB and AP1 activation by TLR ligands and NFAT activation by P2R ligands). Moreover, agents classified in different categories produced distinct "signatures" in DC in terms of gene expression profiles, cytokine and chemokine profiles, and surface phenotypes. These results unveil a mechanism by which DC acquire striking plasticity upon exposure to different stimuli. Our DC-based biosensor system should serve as a useful platform to dissect the DC activation process and to discover novel vaccine adjuvants and immunotherapeutics that selectively activate desired transcription regulatory pathways in DC.

0877

Correlation between changes in serum high-molecular weight melanoma-associated antigen levels and clinical outcome in melanoma

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There is a need for improved markers to monitor the course of malignant melanoma and its response to therapy, as the time required to reach conventional end-points of disease recurrence and overall survival considerably increases the time and expense required to evaluate new therapies. This study was conducted to examine whether treatment associated changes in levels of the high-molecular weight mlanoma-associated antigen (HMW-MAA) are related to clinical outcome. We measured serum levels of the HMW-MAA in 96 patients with resected AJCC Stage IIb or IIIa melanoma before and following therapy with a polyvalent, shed antigen, melanoma vaccine using a double-sandwich ELISA. The normal range was determined utilizing sera of 33 healthy individuals. Assay for HMW-MAA was quantitative and was reproducible. Elevated baseline levels of HMW-MAA were detected in 31% of patients. Following vaccine therapy, 12 (12.5%) patients had a decrease in antigen level and 11 (11.5%) an increase. Median 75% percentile recurrence free survival RFS was significantly longer in patients whose HMW-MAA level decreased compared to those whose antigen level increased i.e., 23.2 months vs. 3.5 months (p=0.006). Melanoma recurred within 12 months in 73% of patients with increasing HMW-MAA levels compared to 25% of those with decreasing levels (p=0.02). In the remaining 73 (76%) patients HMW-MAA was negative at both time points or remained positive without significant change, and their median 75% RFS in patients was 8.6 months. These results suggest that changes in serum level of HMW-MAA may provide an early marker of the progression and/or response to therapy of malignant melanoma.

0878

Expression of IP-10 is strongly induced during mouse Langerhans cell maturation and its production is differentially regulated by various stimuli compared with that of TARC and MDC

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Th1 and Th2 cells express different types of chemokine receptors. CXC chemokine receptor 3(CXCR3) is predominantly expressed on Th1 cells, and its ligand IFN-inducible protein-10(IP-10) promotes migration of Th1 cells. On the other hand, CC chemokine receptor 4(CCR4) is predominantly expressed on Th2 cells, and its ligands thymus and activation-induced chemokine(TARC) and macrophage-derived chemokine(MDC) promote migration of Th2 cells. It is well known that IP-10, TARC and MDC are produced by antigen presenting cells(APC) including dendritic cells(DC). Langerhans cells(LC) are APC localized in epidermis, and they belong to DC lineage. It is reported that LC express TARC and MDC during maturation. However, it is not well documented whether LC can express IP-10 and how the production of these Th1/Th2 chemokines is modulated by various stimuli in LC. Recently, using the panning method, we succeeded in isolating highly purified LC(>95%) from BALB/c mouse skin. In this study, mRNAs of freshly isolated LC and cultured LC were analyzed by RT-PCR to detect the mRNA expression of these Th1/Th2 chemokines. Second, to examine the regulation of the production of these chemokines, the amount of each chemokine was measured by ELISA using the supernatant of 48h cultured LC with or without various stimuli. We show here that: (1) mRNA of IP-10, as well as TARC and MDC, is strongly induced during maturation in mouse LC. (2) Production of TARC and MDC in LC is either greatly enhanced or inhibited according to the various kinds of stimuli used, but only limited kinds of stimuli can regulate the production of IP-10. These results indicate that IP-10-CXCR3 system is operative only in special microemvironment compared with TARC/MDC-CCR4 system in the skin immune system.

0879

Elevated serum cutaneous T cell-attracting chemokine (CTACK/CCL27) levels in patients with atopic dermatitis and psoriasis vulgaris

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Both atopic dermatitis (AD) and psoriasis vulgaris (PsV) are characterized as chronic and relapsing inflammatory skin diseases associated with various immunologic abnormalities. CTACK/CCL27 (cutaneous T cell-attracting chemokine) is a member of the CC chemokines and a functional ligand for CC chemokine receptor 10 (CCR10). The epidermal keratinocyte is a main source of CTACK, suggesting the involvement of various inflammatory skin diseases. The purpose of this investigation was to clarify whether or not CTACK produced by keratinocytes is detected in sera of patients with AD and PsV and to examine the correlation between the serum CTACK levels and disease activity of patients with AD and PsV. We measured the serum CTACK levels in 50 patients with AD, 30 patients with PsV and 22 healthy control subjects. We also divided 50 patients with AD into three groups; mild, moderate and severe, and compared them among three categories. Moreover, we compared the serum CTACK levels of patients with AD and PsV with clinical or laboratory data. Immunohistochemical staining of CTACK and IP-10/CXCL10 was performed in the lesional skin of patients with AD and PsV. The serum CTACK levels in patients with AD and PsV were significantly higher than those in healthy control subjects. The serum CTACK levels in patients with AD significantly correlated with SCORAD, serum sIL-2R, sE-selectin, TARC and MDC levels. Those in patients with PsV significantly correlated with the serum IP-10 levels, but not with the PASI score. Immunohistochemical staining showed CTACK was strongly expressed in lesional keratinocytes of patients with AD and PsV whereas IP-10 was strongly expressed in those of patients with PsV and focally in those with AD. These results suggest that CTACK may be one of the important chemokines for the pathogenesis of AD and PsV.

0880

T helper type 2-dominated response in lck-GATA-3-transgenic mice up-regulates the contact hypersensitivity reaction

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Contact hypersensitivity is thought to be mainly associated with the activation of Th1 cells. However, there is also evidence that Th2 cells and Th2 cytokines play a role in the development of contact hypersensitivity. In the present study, we set out to determine the Th2 response in contact hypersensitivity reaction to 2,4,6-trinitrochlorobenzene (TNCB) in lck-GATA-3-transgenic mice. TNCB-challenged lck-GATA-3-transgenic mice had significantly greater ear-swelling responses than TNCB-challenged wild-type (wt) mice. The dermis in the challenged skin in lck-GATA-3-transgenic mice was the very severe inflammatory response with the lesional inflammation of both eosinophils, monocytes/macrophages and mast cells compared with wt mice. The expression of Th2 cytokines (IL-5 and IL-13) in TNCB-challenged skin tissues and the IgE response after chalenge were profoundly increased in lck-transgenic mice, whereas the expression of interferon-γ was the same in lck-GATA-3 trasgenic mice and wt mice after challenge. TNCB-challenged lck-GATA-3 transgenic mice appeared to mimic the reaction of Th2 cells and Th2 cytokines occurring within the lesional skin in atopic dermatitis (AD) patients. These results suggest that GATA-3-induced Th2-dominated responses play a critical role in the pathogenesis of allergic dermatitis such as AD.

0881

Epidermal Langerhans' cell migration in psoriasis

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Epidermal Langerhans' cell (LC) migration is in part dependent upon signals provided by tumour necrosis factor-a(TNF-a). For instance, intradermal administration of homologous recombinant TNF-a results in a significant reduction (approx 25%) in LC numbers in normal epidermis within 2 hours of treatment. By contrast the characteristics of epidermal LC migration in psoriasis are poorly understood. Thus we examined the migration of epidermal LC in patients with a range of severities of chronic plaque psoriasis following exposure to TNF-a. 4 healthy volunteers (20-55 years) and 8 patients with psoriasis (27-53 years), each received 50 249.3 LC/mm2; mean ± SD for n=4 normal subjects and 1055.2 ± 125.7 LC/mm2; mean ± SD for n=8 psoriasis subjects). However, whereas TNF-a provoked a marked reduction in CD1a+ LC numbers in normal subjects at both concentrations tested (mean reduction 27% for 200U, n=2, and 24% for 500U, n=2), TNF-a failed to stimulate LC migration in patients with psoriasis (mean reduction 0.8% for 200U, n=5; and 1.0% for 500U, n=3). In 4 other patients with psoriasis (27-52 years), LC frequency was measured as a function of HLA-DR expression. HLA-DR+ LC densities at saline treated sites were within the range of values for control sites stained for CD1a (989.9 ± 273.1 LC/mm2; mean ± SD) and no change (mean reduction: 0.05%) in LC numbers was observed in response to 500U TNF-a. These results suggest that in psoriasis there is a functional abnormality in the epidermal LC population that manifests as a lack of migratory response to TNF-a.

0882

Adaptive immunity relies on distinct B cell recruitment for antigen presentation in murine leukocyte adhesion deficiency 1

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Leukocyte adhesion deficiency type 1 (LAD1) is a severe primary immunodeficiency syndrome caused by a lack of functional β_2 integrins. LAD1 patients suffer from recurrent bacterial infections, impaired wound healing and skin ulcers. Weak or absent vaccine responses are concurrent. We investigated the role of β_2 integrins in adaptive immune responses towards T-dependent antigens in β_2 integrin-deficient ($CD18^{-/-}$) mice. Immunohistochemistry and ultrastructural analyses revealed a deficiency in forming morphologic corner stones of secondary lymphoid tissue such as germinal centers and immunological synapses. Upon immunization with tetanus toxoid (TT), $CD18^{-/-}$ mice generated only weak primary and secondary IgG responses, paralleling reports on human and bovine LAD1. To specifically amplify the capacity of antigen presentation, $CD18^{-/-}$ and wild-type mice were immunized with densely haptenated, (4-hydroxy-3-nitrophenyl) acetyl- (NP-) coupled antigens (NP-chicken γ globulin, NP-TT). Despite pronounced morphological alterations, and the failure to respond to TT, we found that $CD18^{-/-}$ mice mounted full primary and secondary immune responses when immunized with haptenated antigens. These were not dependent on the prevailing high interleukin-6 (IL-6) serum concentrations in $CD18^{-/-}$ mice, as could be demonstrated by the generation of a $CD18^{-/-}$ $IL\text{-}6^{-/-}$ mutant. Since haptens have the distinct ability to recruit additional B cells with hapten specificity into antigen presentation, they thus increase the pool of antigen presenting cells. This is crucial for the successful elicitation of adaptive immune responses in LAD1. These findings may argue in favour of a modification of vaccination strategies in immunodeficient individuals.

0883

Prevention of generalized autoimmune skin disease by transferring regulatory T cells

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We established a model mouse of spontaneous generalized autoimmune skin disease mimicking toxic epidermal necrolysis (TEN) affected by extrathymically developed cytotoxic T lymphocytes (CTL). Double transgenic mice carrying both the transgene of ovalbumin (OVA) expressed selectively in the keratinocytes under the keratin 5 (K5) promoter (K5-mOVA) and the Kb-restricted OVA-specific TCR transgene(OT-I) spontaneously developed TEN-like skin phenotype in athymic nude mice background. In contrast, the double transgenic mice in euthymic mice background never developed the skin phenotype. The number of OT-I cells was extremely decreased in euthymic double transgenic mice because of clonal deletion by OVA-expressing thymus in which K5 was active. However, when the OT-I cells purified from the lymph node of euthymic double transgenic mice were transferred into K5-mOVA single transgenic athymic nude mice, they vigorously attacked the skin bearing OVA antigen, resulting in TEN-like skin damage. These results strongly suggested that tolerance of OT-I cells might be induced by thymus-derived cells in the euthymic mice. To demonstrate the existence of tolerogenic immune system, we transferred CD8 (-) lymph node cells of euthymic double transgenic mice into athymic double transgenic mice at day 10 of age. Although athymic double transgenic mice usually developed severe skin disease at 8-12 weeks of age, transferred mice were completely rescued from skin phenotype at more than 24 weeks of age. Moreover, depletion of CD4 (+) cells from inoculating cells resulted in failure of tolerance induction in athymic mice. These results indicate that so-called regulatory T cells strictly prevent the cytotoxicity of OT-I cells in euthymic double transgenic mice, and that antigen expressed under control of K5 promoter in the thymus not only induces clonal deletion but also generates regulatory T cells preventing autoimmune skin disease.

0884

Priming with formaldehyde-treated tumor cells broke tolerance and induced anti-tumor immune response in murine melanoma models

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Tumor antigen-specific immune responses are often not evoked in tumor-burden mice. At first tumor antigens induce specific immunologic tolerance in lymph organs. In addition, the products of tumor cells may suppress immune responses of antigen presenting cells (APC) and/or lymphocytes. Therefore, we modified murine melanoma cells, B16F1, to create the following: (1) 1% formaldehyde-treated cells (fixed), (2) sonicated fixed cells, and (3) frozen and thawed cells (F&T). We then determined which modified tumor cells elicited the strongest anti-tumor immunity. C57BL/6 mice were intradermally immunized three times at one-week intervals with the modified melanoma cells before or after challenge with syngeneic melanoma. Some mice were transfected with 6μg IL-12 p35/p40 expression plasmid DNA (IL-12) into the skin around the tumor-immunization site with the gene gun. In the preventative assay, in over 60% of the mice primed with fixed tumor followed by IL-12 administration, tumor establishment was completely rejected. In the therapeutic assay, tumor size decreased in mice primed with fixed tumor but not in those primed with sonicated fixed tumor. IL-12 administration enhanced anti-tumor immunity both in mice primed with fixed and sonicated fixed tumor compared with mice only administered IL-12. The lymphocyte depletion assay demonstrated that both CD4+ and CD8+ T cells played critical roles in the protection against melanoma. In this experiment, melanoma-specific proliferation in lymphocytes was enhanced only in mice primed with fixed tumor. In conclusion, priming with fixed tumor cells can elicit significant anti-tumor immunity both in preventative and therapeutic models. The activation of dendritic cell might be critical to this enhanced anti-tumor immunity, because fixed melanoma cells should be phagocytosed, processed and antigen-presented by APC as non-treated cells. Fixed tumor cells can be a potential antigen source in several immune therapeutic strategies against tumors.

0885

CD4+CD25+ regulatory T cells and the regulation of cutaneous antigen-specific inflammation in humans *in vivo*

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The resolution of antigen-specific inflammation is dependent on mechanisms to switch off the immune response. CD4+CD25+ regulatory T cells exhibit suppressive activity and are anergic. Evidence suggests that regulatory T cells are highly differentiated cells with short telomeres and low expression of CD45RB. We have used the Mantoux test (MT) to investigate the role that regulatory T cells may play in controlling an immune response *in vivo*. CD4+ T cells isolated from the peripheral blood (PB) and skin suction blisters raised over MTs have been analysed. Antigen-specific telomere length was measured using a novel 3-colour flow FISH technique. The clinical peak of the MT is at day 3 and is associated with a low proportion of antigen-specific CD4+ T cells (1.6%). The peak of infiltrating T cells, however, is at day 7 and is marked by a significant increase in antigen-specific cells (12%). This increased to 27% by day 21. PPD-specific CD4+ T cells isolated from the skin at day 21 had shortened telomeres compared to PB PPD-specific cells (MFI 85 vs 152 respectively). Significant shortening was not detected at days 3 and 7. CD45RB expression was also significantly decreased in skin memory CD4+ T cells by day 21. This points to the on-going differentiation of cells within the skin, which results in a highly differentiated antigen-specific population of cells phenotypically similar to regulatory T cells. There was, however, no accumulation of CD4+CD25+ T cells during the course of the MT and no change in the proportion of PPD-specific cells capable of IL-2 secretion. Furthermore, CD4+ T cells isolated from the skin during resolution were hyperproliferative compared to PB CD4+ T cells and did not suppress PB CD4+CD25- T cell proliferation. Lack of significant suppressive activity at day 21 suggests that regulatory T cells do not play an important role in the resolution of cutaneous inflammation.

0886

Improved responsiveness of Langerhans/dendritic cells to allergens

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Langerhans cells (LC) are immature dendritic cells (DC) that are highly specialized antigen-presenting cells (APC) located in the skin, mucosa, and lymphoid tissues. LC play a key role in the induction phase of contact allergenicity, and hence it may be possible to develop an in vitro LC-based assay for contact sensitization. The difficulty in harvesting and the short survival time of LC in culture have prevented researchers from widespread experimental use of LC. Although improvements have been made, generating large number of cells remains a limiting factor and the functionality and cytokine production capacity of cells in response to different stimuli is not consistent. Here we report a new method of generating LC from CD34+ progenitor cells harvested from umbilical cord blood. The generated LC were expanded 200 fold and expressed CD1a and HLA-DR, characteristic of LC. LC were cultured for up to 41 days with no significant changes in CD1a and HLA-DR expression. Transmission electron microscopy showed the presence of Birbeck granules, a key ultrastructural marker of LC. Upon stimulation with lipopolysaccharide and phorbol 12-myristate 13-acetate, the LC showed a reproducible (n = 4), high level of gene and protein responsiveness in terms of IL-12, MIP-1α, MIP-3α, IL-6, and TNF-α expression. The generated cells were infectable with HIV and they were able to stimulate allogeneic T cells. In conclusion, we have developed a method to harvest and culture functional LC expressing key LC markers. These cells have longer life span in culture and can be used in: 1) allergenicity, 2) viral infection, 3) antigen presentation, 4) immuno-therapeutic, and numerous other studies.

0887

Telomere shortening and down-regulated telomerase activity during resolving cutaneous T cell-mediated inflammation in humans *in vivo*

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The Mantoux test (MT) is a CD4 T cell mediated recall immune response that represents an excellent model of resolving cutaneous inflammation in humans. The skin suction blister technique has allowed us to characterise the course of MT response and follow the on-going differentiation of cells in humans *in vivo*. One consequence of T cell proliferation and differentiation is telomere shortening. We have previously established that the MT peaks clinicllly at day 3 while the peak of infiltrating T cells and proliferation occurs at day 7. Cells were collected from blisters at various timepoints during the induction, peak and resolution phases of the response and compared to cells derived from peripheral blood. We observed an increase in the proportion of highly differentiated CD45RBlow memory CD4+ T cells in the skin at day 21 compared to blood (65% and 87% respectively, $p<0.001$). PPD-specific CD4+ T cells isolated from the skin at day 21 had significantly shorter telomeres compared to PPD-specific cells in the blood (telomere MFI 85 vs 152 respectively). Heteroduplex analysis confirmed the presence of the same clones throughout the response. This indicates that proliferation and differentiation of CD4+ T cells occurs in situ during the MT. The shortening of telomeres observed within the antigen specific population of CD4+ T cells in the skin may be explained by a low level of telomerase activity situ. Our data suggests that telomerase activity is only present transiently early in the response. At the peak of proliferation at day 7 telomerase activity was at undetectable levels. Our preliminary data suggest that the lack of telomerase function may be explained by the increased levels of interferonα observed in blister fluids at day 3.

0888

Ligation of CD86 increases dendritic cell capture of naive T cells and up-regulates CD40 and MHC II expression

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CD86 is expressed by maturing DC, including Langerhans cells, and modulates T cell function when it binds to CD28 and CTLA4 on T cells, but the effects of CD86 ligation on DC are unclear. Herein, we address whether CD86-mediated signaling modulates DC function and surface receptor expression. To crosslink CD86 (or other cell surface markers for comparison), murine bone marrow-derived DC (BMDC) were added to tissue culture plates that had been coated with anti-CD80, anti-CD86, or control monoclonal antibodies (mAb). After 12 hr at 37 deg C, BMDC were then analyzed by flow cytometry for CD40 and MHC class II expression. 51% of CD86-ligated BMDC had a fluorescence intensity (FI) for CD40 of >800, compared with only 0.62% and 2% for anti-CD80 and isotype mAb-treated BMDC, respectively. 47% of CD86-ligated BMDC had an FI for MHC Class II above 6000, compared with 16% of CD80-ligated and 3% of isotype mAb-treated BMDC. By quantitative RT-PCR analysis, ligation of CD86, but not CD80, increased mRNA expression of the T cell chemoattractants, CCL5 (RANTES) and CCL17 (TARC), by 5 and 4.2 fold, respectively, compared to isotype mAb. To evaluate DC adhesion to T cells, naive T cells isolated from mouse peripheral LN were introduced into a parallel plate flow chamber containing BMDC that had been immobilized on anti-CD80, CD86, and CD40 mAb-coated tissue culture plates for 2 hr at 4 deg C and then 1 hr at RT prior to assay. Interestingly, anti-CD86-immobilized BMDC captured naive T cells two-three fold better than BMDC immobilized by CD40 or CD80 mAb ($p<0.0001$). DC from mouse skin explants showed similar enhancement of T cell binding after CD86 ligation. Thus, in vitro engagement of CD86 on DC up-regulates DC maturation markers, chemokines that can attract T cells, and DC capture of T cells. Interestingly, these effects were not seen when CD80 (another member of the B7 family) was crosslinked. These results suggest that engagement of CD86 on DC may play a selective role in regulating DC-T cell binding and antigen presentation in vivo.

0889

The malignant cells of cutaneous T cell lymphoma retain properties of the immunoregulatory precursors from which they derive and are responsive to common CTCL antigens

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We have demonstrated that the growth of cutaneous T cell lymphoma (CTCL) is driven by class II presentation of peptides endogenous to the CTCL cells themselves. CTCL cells stimulated by auto-antigen develop a T-regulatory (T-reg) phenotype. We now demonstrate that this antigen, when presented by autologous dendritic cells (DC), induces a T-reg phenotype in both allogeneic CTCL cells and normal CD4 T cells. CTCL cells, DC and normal CD4 T cells were expanded with GMCSF/IL4 and IL2/IL7. Purified CTCL cells were rendered apoptotic by incubation with CD3 antibody prior to being internalized by autologous or allogeneic DC. The phenotype of CTCL cells or normal CD4 T cells, after addition to autologous pulsed DC, was assessed. Purified CTCL cells that adopted a T-reg phenotype were added as third-parties to measure suppression of a normal control mixed leukocyte culture (MLC). After stimulation by tumor-loaded DC, CTCL cells or normal control CD4 T cells expressed a T-reg phenotype (>20% membrane CD3, CD4, CD25+/cytoplasmic CTLA4+). In contrast, neither CD4-treated CTCL cells, normal CD4 T cells, autologous DC alone, nor unpulsed allogeneic DC induced expression of a T-reg phenotype (<5% + cells). Adoption of a T-reg phenotype depended on the concentration of CTCL-loaded DC (38% CTLA4+/10^5DC) and the number of apoptotic CTCL cells used to load the DC (10^6 CTCL cells:38% CTLA4+). CTCL cells that adopted a T-reg phenotype were functional third-party suppressors of a normal MLC response (51% T-reg CTCL mediates 63% MLC suppression). These results suggest that CTCL is a malignant expansion of a normal clone of T-reg cells. The common CTCL antigen(s) is recognized by autologous, and allogeneic CTCL cells, as well as normal CD4 T cells. Since the antigen is endogenous to CTCL cells from multiple sources, but not normal CD4 T cells, it may reflect a protein with an etiologic relationship to the malignancy.

0890

Allergen-DNA-transfected dendritic cells as a tool to influence the human allergic immune response

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Atopic/allergic diseases are characterized by TH2 dominated immune responses resulting in IgE production. DNA-based immunotherapies have been shown to shift the immune response towards TH1 in animal models. The aim of the study was to analyze wether DC transfected with allergen-DNA are able to stimulate human autologous CD4+ and/or CD8+ T cells from atopic individuals to produce TH1 cytokines instead of TH2 cytokines. For this purpose, human mature DC from atopic donors were transfected with an adenovirus encoding the allergen Phl p 1. Autologous CD4+ and CD8+ T cells were stimulated with these transfected DC and proliferation and cytokine production were measured. Using an adenoviral vector a transfection rate of 92% could be achieved. The proliferative response of CD4+ T cells stimulated with autologous transfected DC was dose dependent and almost as high as that of T cells stimulated with mature allergen pulsed DC. The proliferation of CD8+ T cells stimulated with transfected DC however, was higher than that of cells stimulated with allergen pulsed DC. The cytokine pattern showed a shift towards a TH1 immune response compared with T cells stimulated with allergen-pulsed DC. Human DC can be transfected with allergen-DNA very efficiently using an adenoviral vector yielding DC with high T cell stimulatory capacities directing the atopic/allergic immune response from TH2-dominance towards TH1.

0891

A TLR2-ligand induces pro-inflammatory cytokines and release of an antimicrobial peptide in human mast cells: role of mast cells in innate immunity of the skin

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Mast cells (MC) have for decades been viewed as effector cells in the immune response associated with Th2 cells and IgE, including urticaria and atopic dermatitis. However, recent studies in mouse models have shown that mast cells express TLR-receptors and can contribute significantly to the innate immune response against infectious agents. MC can rapidly respond to the infectious microorganisms, by releasing immunoregulatory mediators, cytokines and recruiting neutrophils. We sought to investigate if human MC play a role in the innate immunity of the skin, using leprosy as a model. We found numerous mast cells in the skin lesions of the leprosy patients. A human MC line (HMC-1) express TLR2 and can be activated by 19kD mycobacterium lipopeptide, releasing pre-formed TNFα and IL-8. Cathelicidin - a novel antimicrobial peptide - was detected by real-time PCR and confirmed at the protein level by FACS analysis and confocal microscopy after TLR-ligation in the HMC-1 and in the lesions of the leprosy patients. We also found that stimulation of the HMC-1 with the 19kDa lipopeptide, upregulates the expression of TLR1, a TLR that forms heterodimers with TLR2 and can confer unique ligand recognition patterns in comparison to TLR2 alone. Our data indicate that MC can be activated via pattern recognition receptors to increase antimicrobial mediators and suggest that MC can contribute to the antimicrobial host response in the skin.

0892

Four-dimensional (x,y,z, time) imaging of Langerhans cell movement in living tissues

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Langerhans cells (LC) are strategically located at the environmental interfaces (e.g., skin and cornea), where they serve as primary Ag presenting cells. Although LC migration from these tissues to draining lymph nodes have been documented by immunohistochemical analyses of fixed specimens, actual behaviors of LC in living tissues remain unknown. In this study, we have created chimeric mice by injecting bone marrow cells isolated from the green fluorescence protein (GFP) transgenic mice into lethally irradiated B6 recipient mice. When tested 2-3 months after bone marrow reconstitution, >90% of IA^+ dendritic cells in the epidermis and the cornea epithelium expressed GFP. Conversely, >80% of GFP^+ dendritic cells expressed LC markers (i.e., IA and DEC205) in both tissues. Ear skin and corneal samples harvested from the chimeric animals were then examined by multi-photon confocal microscopy (Leica SP2/Coherent Ti:Sapphire femtosecond pulsed laser). Tissue was mounted on a tissue slice adapter with temperature and media flow rate control. After tissue stabilization (~1 hr), 3D data sets were collected every 2-5 min for 1-3 hr. Images were then reconstructed to evaluate cell movement in xyt, xzt and yzt, simultaneously. The time lapse confocal analyses revealed striking mobility of GFP^+ LC in both epidermis and cornea. Virtually all GFP^+ cells showed rhythmic and synchronized movement of their dendrites, primarily composed of repeated projection/retraction cycles with an interval of 33 ± 5 min/cycle and speeds of 1.5 ± 0.2 μm/min (projection) and 2.0 ± 0.6 μm/min (retraction). Some (~5%) of the GFP^+ cells showed rapid migration through the epithelial layer at a speed of 4.6 ± 3.8 μm/min with snaking of dendritic processes around and between adjacent keratinocytes. Some vertical migration through the lamina propria was also noted. This is the first report showing real-time 3D images of LC movement in living tissues and unveiling a peculiar dendrite projection/retraction cycle.

0893

Keratinocyte extract enhances IgE production and epsilon germline transcription *in vitro*

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[Objective] Serum IgE level of atopic dermatitis patient is correlative to the severity of skin lesion. We hypothesized that destruction of keratinocyte enhances IgE production and have reported that serum IgE level of BALB/c mouse increased when injected with PAM212 (keratinocyte cell-line of BALB/c mouse) extract. (Yamamoto T. et al. Arch Dermatol Res 294:117, 2002) Here, we examined the effect of PAM212 extract on IgE production and B-cell class switching *in vitro*. [Method] PAM212 cells were cultured and homogenized by sonication. The soluble fraction was obtained by centrifugation (105,000 *g*)(PAM extract). Splenic lymphocytes were obtained from BALB/c mouse and cultured with or without IL-4, anti-CD40 antibody, and PAM212 extract. Supernatant IgE level was measured by ELISA. Epsilon germline transcription in cultured cells was measured by semi-quantitative RT-PCR from mRNA. [Result] PAM extract increased supernatant IgE level and enhanced epsilon germline transcription in dose-dependent manner. Both IL-4 and anti-CD40 antibody were required for these effects of PAM extract. [Conclusion] PAM extract enhances IgE production by splenic lymphocytes via the enhancement of class-switching to IgE.

0894

Primary lymphocyte activation during induction of contact hypersensitivity is potently inhibited in mice by tacrolimus but not by pimecrolimus

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Contact hypersensitivity (CHS) is a T cell-mediated, antigen-specific dermatitis which is elicited in sensitized individuals by epicutaneous exposure to the antigen. Oral pimecrolimus (PI) and oral tacrolimus (TA) have been shown to inhibit the elicitation of CHS with similar activity in mice sensitized against oxazolone. Interestingly, the induction phase (sensitization) associated with hyperplasia of local lymph nodes (LLN) was inhibited significantly by TA only, even at one third of the highest PI dose tested. This striking difference prompted us to analyze phenotypes and kinetics of activation-associated surface markers of lymphocytes of LLN. BALB/c mice were treated orally with 30 mg/kg TA or 90 mg/kg PI 2 hrs before, 4, 24, and 48 hrs after sensitization and dissected 48 or 96 hrs after sensitization. LLN cells were prepared and subjected to analysis by flow cytometry. LLN from placebo-treated sensitized mice (controls) and from normal mice were prepared similarly for comparison. At 48 hrs (24 hrs after the third dosing) a higher proportion of LLN cells from controls expressed activation antigens such as CD25 (5.6%), CD69 (41.7%) and CD134 (9.6%) than in normal mice. TA significantly decreased the number of activated lymphocytes bearing these markers (2.7, 21.2 and 5.3%, respectively) whereas the changes by PI vs controls were not statistically significantly different. At 96 hrs (48 hrs after the fourth dosing), 41.7% of all LLN cells were B cells (B220+/CD19+) in controls vs 25.6% in normal mice. B cells showed an activated phenotype as assessed by double staining for B220/CD95 (41.7%) and B220/CD40 (39.7%). TA, in contrast to PI, significantly reduced the number of activated B cells as shown for B220/CD95 cells (TA:30.7% vs PI:41.7%) and for B220/CD40 (TA:29.3% vs PI:42.1%). Taken together, our results indicate that treatment of mice with TA, opposed to the treatment with PI, results in a potent inhibition of lymphocyte activation in LLN during sensitization.

0895

ATP enhances cutaneous immunity by modulating Langerhans cell function

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ATP can signal through cell surface receptors that are either ionotrophic (P2X) or G-protein linked (P2Y) and ATP has been shown to modulate some aspects of dendritic cell function. Langerhans cells (LCs) are a class of dendritic antigen presenting cells that reside in the epidermis and are believed to play an important role in the initiation and expression of cutaneous immune responses. The response of LCs to ATP has not been studied. In this study, we have examined the effects of ATP on functional properties of LCs using the murine LC-like cell line, XS106A, derived from neonatal A/J (H-2^a) epidermis as a surrogate for LCs. XS106A cells were cultured in graded concentrations of ATP. ATP (10 μM to 1000 μM) augmented cell surface expression of CD80 and CD86 at 24 hrs and increased expression of I-A at 48 hrs as measured by flow cytometry. Culture in ATPγS, a stable analogue of ATP, modified cytokine expression. It significantly enhanced secretion of IL-1β induced by lipopolysaccharide, but significantly decreased secretion of IL-10, as measured by ELISA. We then examined whether exposure to ATPγS would modulate antigen presenting ability. Culture in ATPγS (1 μM to 1000 μM) followed by thorough washing significantly augmented the ability of BALB/c (H-2^d) epidermal cells to present antigen (KLH) to a responsive T cell clone [HDK1 (H-2^d)]. To test whether ATP might also enhance cutaneous immunity in vivo, we studied the effect of ATPγS on contact hypersensitivity (CHS). Intradermal injection of ATPγS significantly enhanced induction of CHS to dinitrofluorobenzene applied topically at the injected site. Finally, by RT-PCR, XS106A cells were found to express mRNA for both P2X and P2Y receptors. These data suggest that local production of ATP, and possibly other nucleotides, may play an important role in regulating the ability of the epidermis to initiate or express an immune response through interaction of nucleotides with a specific receptor(s) on the surface of resident LCs.

0896

Epinephrine regulates TNF-α expression in the Langerhans cell-like cell line XS106A

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Epidermal Langerhans cells (LC) express adrenergic receptors and agonist-binding to these receptors regulates LC function. Exposure of purified LC to epinephrine (EPI) and norepinephrine (NE) in vitro inhibits presentation of a defined antigen to a responsive T cell clone. They also inhibit elicitation of delayed-type hypersensitivity by epidermal cells in a β-receptor-mediated fashion, and intradermal injection of EPI inhibits the induction of contact hypersensitivity. To examine possible mechanisms for these activities, we studied the effect of EPI on LPS-induced expression of mRNA for IL-12 by semiquantitative RT-PCR as well as production of TNF-α, IL-1β, IL-10, and IL-12 protein (assessed by ELISA) by the LC-like cell line XS106A . Cells were plated at 10^6/ml and incubated with or without EPI 3 h prior to stimulation with 0.1 mg LPS. In a subgroup of experiments, cells were incubated with the β-adrenergic antagonist propranolol or the α-adrenergic antagonist phentolamine 1 h prior to EPI treatment. Without LPS-stimulation cells did not produce detectable levels of TNF-α. In supernatants collected 3h after addition of LPS, TNF-α was strongly upregulated [225+/-31 (SD) pg/ml]. This effect was significantly inhibited by pretreatment with EPI 10^{-7} M (157+/-21 pg/ml). Propranolol 10^{-6} M prevented the EPI-induced inhibition (233+/-15 pg/ml), while phentolamine had no effect (173+/-8 pg/ml). In 24 h supernatants, IL-1β and IL-10 production was upregulated after stimulation with LPS. Preliminary data suggest that EPI may reduce the upregulation of IL-1β while it may increase the production of IL-10. Preliminary experiments indicate an upregulation of IL-12 mRNA expression 8h after addition of LPS and protein production 24 h after LPS addition, both of which are also inhibited by EPI. Our findings indicate that catecholamine-induced suppression of cutaneous immunity may be mediated, in part, by changes in cytokines secreted by LC upon stimulation.

0897

Hapten application induces the production of IL-16 by epidermal cells in induction and elicitation phase of contact hypersensitivity reaction

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Interleukin (IL)-16 is a chemoattractant cytokine for CD4 positive leukocytes and is synthesized by a variety of immune (T cells, eosinophils, and dendritic cells) and non-immune (fibroblasts, epithelial cells, and neuronal cells) cells. Because contact hypersensitivity reaction is mediated by T helper 1 cells, we have investigated the involvement of IL-16 in the contact hypersensitivity reaction in which CD4 positive T cells may be chemoattracted by IL-16. Trinitrochlorobenzene was applied to the skin of male BALB/c mice and the expression of IL-16 was estimated at various times. ELISA analysis exhibited the release of IL-16 from the epidermis and the dermis in elicitation phase in a dose-dependent fashion. Interestingly, hapten application alone also induced IL-16 production both in the epidermis and the dermis in induction phase. RT-PCR analysis also demonstrated IL-16 mRNA to be detected in the epidermal cells of the mice not only in elicitation phase, but also in induction phase. No IL-16 mRNA was detected in those of control mice applied vehicle alone. Flowcytometric analysis using monoclonal antibody against I-Ad indicated that IL-16 was produced mainly by keratinocytes after single application of trinitrochlorobenzene. These results suggest that IL-16 may play a role in induction and elicitation phase of contact hypersensitivity reaction.

0898

Immunophenotyping of inflammatory cells in skin lesions of atopic dermatitis: more similarities than differences between extrinsic and intrinsic forms

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It is well known that atopic dermaitits is divided into several subgroups-extrinsic, intrinsic or indeterminate types-according to the presence or absence of allergy. Although the previous studies have demonstrated differences in the cytokine profile between the two subtypes, the precise pathogenesis of both extrinsic and intrinsic forms of atopic dermatitis is obscure. To find out the potential differences in the inflammatory micromilieu of both extrinsic and intrinsic atopic dermatitis, we performed immunophenotyping of inflammatory cells in skin of 18 extrinsic and 17 intrinsic type of atopic dermatitis. The results revealed a predominance of CD4+ T cells in the dermal infiltrate with a similar CD4/CD8 ratio between two subtypes. Expression of other T cell markers and epidermal Langerhans cells including CD30 and HLA-DR was also prominent in both types of atopic dermatitis. Although the dermal T cell immunoprofile was simialr between two subtypes, tissue infiltration of EG2+ and MBP+ eosinophil granular proteins was markedly prominent in extrinsic type compared to intrinsic type. Furthermore, lesional eotaxin immunoreactivity was significantly higher in extrinsic subgroup. Our data suggest that although overall inflammatory microenvironment appears to be similar in two subtypes, differences of cytokine productions in the T cell may contribute to the differential degree of tissue eosinophilia in extrinsic and intrinsic form of atopic dermatitis.

0899

Differential *in vivo* cytokine mRNA expression in lesional skin of intrinsic versus extrinsic atopic dermatitis patients using semiquantitative RT-PCR

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A small subgroup of atopic dermatitis (AD) patients with normal serum IgE levels and without specific IgE sensitization has been termed intrinsic type of AD (ADi) as a counterpart to the term extrinsic type of AD (ADe). But, there are neither molecular makers nor clinically diagnostic tools for distinguishing between ADi and ADe. To clarify the pathogenesis and in vivo cytokine micromilieu of ADi patients in comparison with ADe patients, we used semiquantitative RT-PCR to investigate the expression of various cytokines and checked the tissue eosionphil counts in skin biopsies from both types of AD patients. Although there was no significant difference of cellular infiltrates in the lesional skin between ADe and ADi patients, ADe had significantly increased tissue eosinophilia than ADi. Based on our RT-PCR, the expression patterns of cytokines could be categorized into four groups. First group includes IL-5, IL-13 and IL-1β whose levels of mRNA expression were higher in both types of AD patients than non-atopic (NA) and even higher in ADe patients than ADi patients. Second group includes IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 whose levels of mRNA expression were elevated in both types of AD patients without differences between ADe and ADi patients. Third group includes TNF-α whose mRAN expression was more decreased in both types of AD patients than NA. And the fourth group includes IL-6 and TGF-β, which did not show any differences among three groups. Thes current data demonstrate that the expression of cytokine IL-5, IL-13, and IL-1β mRNA and numbers of dermal infiltrating eosinophils are increased in ADe patients, compared to ADi patients.

0900

Natural anti-keratin autoantibody is encoded by germline gene

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Anti-keratin autoantibody(AK autoAb), the circulating autoantibody binding to keratin polypeptide, has been detected in all healthy individuals. Although some progresses have been made in determining its functions and significances, the mechanism of natural AK autoAb production remains an enigma. According to Clone tolerance theory, B lymphocytes with self-reactivity should be deleted. The presence of AK autoAb seems to collide with the clone tolerance theory . The present study aimed at obtaining and analyzing the variable region gene sequence of natural AK autoAb. Keratin purified from newborn mice skin was used to screen hybridomas secreting anti-keratin mAb . Fusions were performed between Sp2/0 cell lines and spleen cells of normal SPF(Specific Pathogen Free) BALB/c mice. Total RNA was isolated from hybridoma and cDNA was synthesized. VH and VK ds-cDNA were generated by PCR using the cDNA as template, two pairs of primers complementary to the cDNA in J or FR1 regions of VH and VK. DNA fragments were sequenceed and aligned to Entrez databases of NCBI. Three hybrydomas strains secreting IgG anti-keratin mAb were obtained and denominated as 2H8, 3B4 and 4G2. The results of immunohistochemistry and Western blot showed that three mAbs could bind to keratin specially. The VH or VK sequences differed extremely from each other except VH of 2H2 and 4G2. The disparity of three VH or VK may be due to the different binding epitope and affinity .Both the VH and VK were homologus to germline gene in databases of NCBI, with the highest identity up to 99%. The nucleotide substitutions may be due to somatic mutation. Because all three VH correspond to J558 family, the usages of VH seem to be restrict to VH1 J558 family. With regard to the JH and JK, quite a few nucleotide substitutions could be found when compared with JH and JK germline genes. No suitable germline gene matched DH . These results strongly indicate variable region genes of natural AK autoAb are derived from germline genes . It will be helpful to elucidate the mechanisms of natural AK auto Ab production.

0901

Pituitary adenylate cyclase activating polypeptide inhibits cutaneous immune function

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Pituitary adenylate cyclase activating polypeptide (PACAP) is present in cutaneous nerves. Previous evidence has demonstrated that cutaneous nerves are closely associated with Langerhans cells (LC) and that LC express mRNA for the PACAP receptors VPAC1 and VPAC2. Previously, we have demonstrated that exposure of murine epidermal cells to PACAP in vitro inhibits the ability of these cells to present antigen to a responsive T cell clone and that PACAP inhibited the induction of contact hypersensitivity when injected intradermally into mice prior to sensitization with a contact sensitizer at the injected site. We have now further characterized the regulatory effects of PACAP on cutaneous immunity. To examine the effects of PACAP on presentation of antigen for induction of delayed-type hypersensitivity (DTH), CAF1 ($H\text{-}2^{a/d}$) epidermal cells were enriched for LC content (eEC) by deletion of Thy 1^+ cells. These cells were then incubated with or without PACAP prior to being pulsed with soluble tumor antigens (TAA) from the S1509a spindle cell tumor ($H\text{-}2^a$). These eEC were then injected into a hind footpad of mice previously immunized against S1509a cells and 24 hr footpad swelling was measured. Mice injected with eEC preincubated with PACAP prior to TAA pulsing showed a significantly decreased DTH response compared to the response elicited by cells not exposed to PACAP [11.9 +/- 4 (SD) mm^{-2} vs 21.3 +/- 7, p=0.002]. To examine possible regulatory effects of PACAP on cytokine expression by LC, we employed the murine LC-like cell line XS106A (derived from neonatal A/J epidermis) as a surrogate for LC. In these cells, PACAP significantly inhibited the LPS and GM-CSF-induced stimulation of IL-1β and IL-12 secretion, while significantly augmenting the production of IL-10. We hypothesize that the effect of PACAP on LC antigen presentation may be due to modulation of cytokine production, resulting in downregulation of cell-mediated immunity.

0902

Sensitization or tolerization by DNTB in humans?

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Dinitrothyocyanobenzene (DNTB) has been reported to tolerize mice to subsequent sensitization by dinitrochlorobenzene (DNCB) so we examined whether DNTB modified subsequent responses to DNCB in humans. To show whether responses to DNCB are altered by prior treatment with DNTB the "reference response" to DNCB was first established in 10 volunteers sensitised by application of the challenge regimen of 4 doses of DNCB (6.25, 8.8, 12.5, 18.8μg) which is itself a moderately potent sensitizing stimulus. Four weeks later the same 4 doses were applied to the other arm and 48h responses quantified as thickness with calipers. The mean response to DNCB (AUC obtained from the sum of responses at each challenge dose) was 5.9±1.02(SEM). To examine whether DNTB could sensitize, the same regimen but with DNTB was used in a second group (N=7). Responses to challenge were uniformly negative so DNTB appeared not to sensitize. To see if priming with DNTB altered subsequent reponses to DNCB, 4 DNTB challenge doses were applied 4 weeks before exposure to the "reference regimen" of sensitization and challenge with DNCB. Responses to the final DNCB challenge were similar to those for the reference group (AUC=4.3±1.6; P>0.05). However, initial priming with a single high dose (35μg) of DNTB followed by the "DNCB reference regimen" resulted in much greater responses to DNCB (AUC 13.4±6.02; N=6). Thus, DNTB appears to act as a weak sensitizer, being unable to prime for responsiveness to itself, but able to prime for augmented responses to DNCB. This result could possibly reflect poor penetration rather than weak inherent sensitizing potency. Therefore, a group previously sensitized and challenged with DNCB were challenged with DNTB which elicited strong responses, comparable to those evoked by DNCB itself. Thus, DNTB can be recognised by DNCB-primed T lymphocytes but it appears poorly able to induce activation of naive T cells. However, in humans, it is not a tolerizer for subsequent reactivity to DNCB.

0903

Vasoactive intestinal polypeptide regulation of cytokine production in the Langerhans cell-like line XS106A

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Vasoactive intestinal polypeptide (VIP) is found in cutaneous nerves and its receptors VPAC1 and VPAC 2 are expressed in Langerhans cells (LCs) and the murine LC-like cell line XS106A, derived from neonatal A/J epidermis. Previous work by our group has shown that neuropeptides released by cutaneous nerves affect LC antigen presentation. In order to determine if VIP regulates cutaneous immune function, we employed XS106A cells as surrogates for LCs and determined the effect of VIP on XS106A cytokine production. XS106A cells were preincubated with varying concentrations of VIP for 3 hours prior to addition of LPS and GM-CSF for stimulation of the cells. Supernatants were collected 24 hours later and analyzed by ELISA for IL-10 and IL-1β production. Since IL-12 levels were not easily detectable by ELISA, the effect of VIP on IL-12 expression was assessed at the mRNA level. Cells were treated as above, but harvested for mRNA 6 hrs after addition of LPS and GM-CSF. Semi-quantitative PCR was utilized to determine changes in IL-12 mRNA expression using GAPDH as an internal control. VIP suppressed the LPS and GM-CSF-induced production of IL-1β at 10^{-6} M [88 +/- 6 (SD) pg/ml for 10^{-6} M VIP vs 106 +/- 5 pg/ml without VIP, p=0.01]. Preliminary data also suggests that VIP at 10^{-6} M inhibits induction of IL-12 mRNA in XS106A cells. In contrast, VIP augmented the secretion of IL-10 in stimulated XS106A cells at concentrations of 10^{-8}, 10^{-7}, and 10^{-6} M (779 +/- 18 pg/ml for VIP 10^{-6} M vs 583 +/- 10 pg/ml without VIP, p<0.001). VIP may regulate cutaneous immune function by modulating LC cytokine expression.

0904

***In vivo* transfection of cis element "decoy" against signal transducers and activators of transcription 6 binding site ameliorates IgE-mediated late phase reaction in atopic dermatitis model mouse**

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Signal Transducers and Activators of Transcription 6 (STAT6) plays a crucial role in the transactivation of IL-4, IL-13 that might be involved in the pathogenesis of atopic dermatitis (AD). We reported here that IgE mediated late phase reaction was significantly reduced in STAT6 deficient (STAT6-/-) mice in AD model mouse induced by intravenous injection of monoclonal anti-DNP-IgE antibody and subsequent skin testing with dinitrofluorobenzene (DNFB). Therefore, we hypothesized that synthetic double-stranded DNA with high affinity for STAT6 could be introduced in vivo as decoy cis elements to bind the transcriptional factor and to block the activation of genes mediating AD, thus providing effective therapy for AD. Treatment by transfection of STAT6 decoy oligodeoxynucleotides (ODN), but not scramble decoy ODN after the sensitization by anti-DNP-IgE antibody, had a significant inhibitory effect on not only STAT6 binding to nuclear but the anti-DNP-IgE antibody induced late phase response. The histological analysis revealed that both edema and the infiltration of neutrophils and eosinophils were significantly reduced in STAT6 decoy ODN transfected mice. To examine the mechanism of the in vivo effect of STAT6 decoy ODN, we employed an in vitro mast cell culture system. After IgE receptor engagement, bone marrow cells tranfected by STAT6 decoy ODN exhibited normal histamine release, but their cytokine release (TNF-a, IL-6) was markedly reduced. Here, we reported that the first successful in vivo transfer of STAT6 decoy ODN to reduce the late phase reaction providing a new therapeutic strategy for atopic dermatitis.

0905

Systemic treatment with anti-SHPS-1 antibody suppresses contact hypersensitivity response via inhibiting Langerhans cell migration at the sensitization phase

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Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) is a member of the signal regulatory protein family, which plays an important role in cell motility and is expressed in myeloid cells including monocytes, macrophages, and dendritic cells. Here we demonstrate that murine epidermal Langerhans cells (LCs) express SHPS-1 and *in vivo* treatment with monoclonal antibodies (mAbs) to SHPS-1 reduced contact hypersensitivity (CHS) response. FACS and immunofluorescent studies using mAbs to SHPS-1 and Ia molecules revealed that Ia-bearing LC express SHPS-1. The *in vivo* treatment with mAbs to SHPS-1 before sensitization, but not before elicitation, reduced CHS reponse to DNFB in C57BL6 mice. We further investigated if anti-SHPS-1 mAbs can inhibit LC migration. First, *in vivo* adminitration of anti-SHPS-1 mAbs reduced LC migration to the regional lymphnodes after FITC application to skin. Second, *in vivo* mAb treatment significantly suppressed a decrease of LC number in the epidermis with hapten application. Third, migration of the dendritic cells expressing Ia molecules from skin explants into the culture media was reduced by the addition of anti-SHPS-1 mAb into the culture media. These results indicates that anti-SHPS-1 mAb inhibited the migration of LC at the sensitization phase and thereby suppressed CHS reponse.

0906

Cutaneous CD25+CD4+ T lymphocytes regulate nickel-specific T cell responses

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A specialized subset of regulatory CD4+ T cells that constituively express the CD25 antigen has been recently characterized. CD4+CD25+ lymphocytes are anergic in vitro when engaged through the TCR, express high levels of CTLA-4 antigen, and suppress T cell responses through a mechanism requiring cell-to-cell contact. CD25+ cells are critical for the maintenance of peripheral tolerance to self-antigens. However, their role in the control of T cell responses against exogenous antigens is still unknown. We found that approximately 20% of CD4+CD25+ T cells purified from peripheral blood of healthy, non allergic donors (n 6), express the cutaneous lymphocyte-associated antigen (CLA), a marker for skin-seeking T lymphocytes. CD4+CD25-, depleted from CD25+ cells, displayed 50-80% increased responses to nickel, compared to the whole CD4+ population, both in terms of proliferation and IFN-γ release. In contrast, the CD25+ fraction was anergic to nickel in 5 out of 6 non allergic individuals, failed to release cytokines, but up-regulated CTLA-4 upon nickel exposure. CD25+ T cells dose-dependently inhibited the proliferation to nickel and the cytokine released by autologous CD4+CD25- T cells. Inhibition was not mediated by IL-10 or TGF-β, and was mostly, but not exclusively, mediated by the CD25+CLA+ population. Punch biopsies taken from the site of negative patch tests to nickel in non allergic subjects, showed the presence of an heavy CLA+CD4+ T cell infiltrate with fewer CD8+ T cells, and a substantial number of CD25+ lymphocytes. T cells isolated from the biopsies contained about 20% of CD25+ cells, failed to respond to nickel in vitro, and strongly inhibited the proliferation of blood CD4+CD25- T cells to the metal. These results indicate that CD4+CD25+ cells can regulate peripheral immune responses to haptens in non allergic individuals, and they may have a role in preventing the development of undesired skin allergies.

0907

Role of CC- chemokines, chemokine receptors and polymorphism in promoter regions of RANTES among patients with atopic dermatitis in Taiwan

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CC- chemokines are potent proinflammatory molecules which play an important role in the pathophysiology of atopic dermatitis (AD) and allergy. We determined the plasma level of CC- chemokines in AD and controls by ELISA and used flow cytometry to evaluate the expression of chemokine receptors on peripheral blood mononuclear cells (PBMC) in both groups. We graded clinical severity of AD according to the SCORAD index. Plasma levels of RANTES, MIP-1 and eotaxin were increased in severe AD patients (n=19), but not in moderate AD (n=32) and mild AD (n=13) compared with normal controls (n=23). MCP-1 level is the samte in both groups. Chemokine receptor CCR3 were overexpressed in patients with severe atopic dermatitis as compared with controls, but not CCR2 and CCR5. In addition, polymorphisms in the promoter region of RANTES have been found and associatecd with overexpression of these chemokines. Different studies showed various roles of RANTES polymorphism in German and Hungarian children with atopic dermatitis. So we have also investigated whether the presence of these polymorphisms was associated with atopic dermatitis in Taiwanese children. Sixty-five atopic dermatitis patiens and twenty six controls of comparable ages were screened for genotype with PCR and direct sequencing- based assay. No significant difference in the frequency of the polymorphisms between atopic dermatitis patients and controls was detected. Our finding suggests augmented production of some CC- chemokines such as RANTES, MIP-1 eotaxin and chemokine receptor CCR3, but no MCP-1, CCR2 and CCR5 correlate with clinical severity of atopic dermatitis. In Taiwanese children, there was no association between -28G and -403 A alleles in the RANTES promoter and atopic dermatitis.

0908

Pollen-associated lipid mediators modulate dendritic cell function leading to a reduced capacity to initiate Th1 responses

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The primary site of exposure to pollen grains is the epithelium of the upper respiratory tract, which is densely populated by immature dendritic cells. We recently demonstrated that pollen grains not only function as allergen carriers but are also a rich source of bioactive lipid mediators. Aqueous extracts from phleum pratense and betula alba pollen (APE) contained predominantly monohydroxylated derivatives of linoleic and linolenic acid. In addition, GC-MS analysis of betulla alba APE demonstrated the presence of phytoprostanes E1, F1, A1/B1. Since DC critically influence the outcome of the ensuing T cell polarization, we investigate the biological activity of APE on immature DC. Human pB monocytes-derived DC displayed morphology and phenotype of immature DC (CD1a+, MHC IImed, mannose receptor+, CD14neg., CD83neg., CD86neg.). Exposure to phleum pratense or betula alba APE induced DC maturation as documented by upregulation of MHC class II, CD83, CD86 and increased allostimulatory activity. APE induced DCs to release significantly less IL-12 p40 and p70, as compared to LPS. In addition, APE inhibited dose-dependently the LPS-induced IL-12 p70 release and IL-12 p40 (but not p35) mRNA levels (real time PCR), while no effect on LPS induced IL-6 release was observed. Consistent with that, APE-activated DCs induced significant less IFN-g production in allogeneic naive CD45RA+, CD4+ T cells. These results suggest that pollen associated lipid mediators (PALMs) act as important regulatory signals that modulate DC function in a fashion that favours a reduced Th1 polarization. C.T.-H. and T.J. contributed equally

0909

A reciprocal interplay of tolerogenic APCs and T suppressor cells accounts for the spread of nickel tolerance *in vivo*

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We have reported that Ni^{2+} ions need to be administered together with adjuvant, such as H_2O_2 (1%), in order to induce de novo sensitization to nickel (Ni) in mice and that tolerance can be induced by a 4-week course of 10 mM $NiCl_2$ in the drinking water. In adoptive transfer assays, as few as 10^2 splenic T cells, containing both $CD4^+$ and $CD8^+$ cells, from orally tolerized donors prevented de novo sensitization to Ni of the recipients. In vitro, these Ni-specific T suppressor (Ts) cells were anergic. Here, we analyzed the mechanism underlying Ni tolerance. We found that 10^3 $CD4^+CD25^+$ T cells from orally tolerized donors failed to transfer Ni-tolerance, whereas, 10^2 APCs, i.e., T cell-depleted spleen cells and even 10^3 purified B cells, which were devoid of DCs, of these donors were perfectly able to do so. This tolerance cannot be explained by a simple passive transfer of Ni. Compared with APCs from untreated donors, the APCs from orally tolerized donors showed a reduced allostimulatory capacity in vitro and displayed a tolerogenic phenotype, namely an increased expression of DEC-205 on DCs, and a decreased expression of CD40 on B cells and DCs. Serial adoptive transfers, using orally tolerized $Ly5.1^+$ donors into two successive sets of $Ly5.2^+$ recipients, revealed a spread of tolerance from donor T cells to host T cells. However, this infectious spread of tolerance required participation of the recipients' APCs. The Ni-specific donor Ts cells apparently modified Ni-presenting APCs of the recipient into tolerogenic APCs which, in turn, induced Ni tolerance in the naive T cells of the recipient. The spread of Ni tolerance from donor Ts cells to host APCs required challenge with both Ni^{2+} ions and H_2O_2, indicating that the Ni-specific donor Ts cells need to be reactivated in order to render the host's APCs tolerogenic. Hence, restimulation of Ni-specific Ts cells not only prevented Ni sensitization of the host, but, paradoxically, enhanced Ni tolerance by the powerful mechanism of infectious tolerance.

0910

Blockade of lymphotoxin signaling inhibits the clinical expression of murine graft-versus-host skin disease

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Early murine GVHSD can serve as an experimental (exp) model for the human cutaneous lichenoid tissue reaction (LTR), a pattern seen in human skin diseases such as lupus erythematosus, dermatomyositis, and lichen planus. As interaction of membrane LTα1B2 (LTα) with its receptor, LTαR, is essential for normal immune functioning, we questioned whether LTα signaling might play a role in the expression of GVHSD. Bone marrow and spleen cells (4 million splenocytes) from 6-8 week-old female LP/J mice (H-2b) were transplanted by retro-orbital vein infusion into a panel of 5 lethally-irradiated 8-12 week-old female C57BL/6 (H-2b) recipient mice. At day 0 and 7 after-transplant (A-T), recipient mice were injected by retro-orbital vein with 75mg of LTBR-Ig, a LTB receptor decoy fusion protein know to inhibit LTα signaling (exp group [exp grp]) or 75mg of human-Ig (control group [cnt grp]). This same exp was replicated twice with similar results. Posterior neck skin biopsies from the cnt grp on day 20 A-T revealed ~ 40% increase in epidermal thickness and ~200% increase in dermal thickness due largely to prominent mononuclear cell (MC) infiltration of the dermis and epidermis. Skin biopsies at the same point in the exp grp demonstrated only low levels of MC infiltration and normal architecture overall. By 40 days A-T, 50% of the cnt grp had developed clinically-obvious, severe skin lesions (les) consisting of varying elements of blistering, erosion, crusting, hyperkeratosis, and alopecia on the face, ears, neck and abdomen. However, the exp grp had not developed clinical skin les at this time point. Analysis of skin and spleen T cells by immunochemistry and flow cytometry revealed that LTBR-Ig treatment inhibited T cell infiltration into skin but and spleen T cell activation. We conclude that inhibition of LTα signaling via LTBR-Ig treatment can ameliorate skin les in murine minor H2 antigen mismatch GVHSD probably by inhibiting T cell activation and homing to skin. The LT signaling pathway could play an important role in the pathogenesis of the human LTR.

0911

Diphenylcyclopropenone sensitization and therapeutic failure in alopecia areata

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The mechanism of action of topical diphenylcyclopropenone (DCP) in the treatment of alopecia areata (AA) and reasons for the highly variable therapeutic successes, are unclear. Because there are few reports of the in vitro immunologic properties of DCP, we explored its effects on peripheral blood mononuclear cells (PBMC) from topically treated donors. Preliminary experiments showed that PBMC responses to DCP were dependent on the presence of a >50 kDa ultrafiltration fraction prepared from fresh autologous serum, but were not enhanced by addition of stratum corneum extract as a source of putative hapten binding proteins. Reversed phase HPLC analysis showed that DCP was converted to a major, non-polar, biologically inactive metabolite by liver and skin microsomes, the latter almost completely converting saturating concentrations of DCP to this metabolite in 4 hours. These preliminary experiments established that 5-day proliferation assays with PBMC, 10% fresh autologous serum and a range of DCP concentrations up to 20 μM (limit of water solubility approximately 200 μM), provide a reliable indication of the level of sensitization to DCP. Analysis of the PBMC responses of patients who had undergone weekly topical DCP treatment of AA for at least 5 months, indicated that DCP-induced PBMC proliferation was significantly greater in the minority of patients who were therapeutic responders (maximum stimulation index [mSI] 13.7 ± 8.6, n=5) than in the therapeutic non-responders (mSI 2.5 ± 1.3, n=9; means ± SD, p=0.009, Mann-Whitney test). Almost 70% of the latter were not sensitized to DCP as shown by the PBMC proliferation assays. In spite of the in vitro immunologic differences, inflammation in treated skin, measured by a defined scoring system, was not different in therapeutic responders and non-responders. These results associate therapeutic failure with failure of DCP sensitization, and suggest that skin inflammation during DCP treatment is not a good measure of DCP allergy. Metabolism by skin microsomal enzymes may also be a mechanism for inactivation of DCP during therapy.

0912

Morphological evidence of neuropeptide release and mast cell degranulation in tacrolimus (FK 506) and pimecrolimus (SDZ ASM 981) treated murine skin

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Topical therapy of atopic dermatitis with the calcineurin inhibitors pimecrolimus and tacrolimus has proven to be a very effective novel approach. However, following tacrolimus and to a lesser extent also after pimecrolimus application many patients experience transient, adverse events such as burning and itching. Since the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are the major cutaneous mediators of pain and pruritus the effect of topical calcineurin inhibitors on neuropeptide release was investigated. Healthy Balb/c mice were treated on the shaved back twice daily with either 0.1% tacrolimus ointment or 1% pimecrolimus cream for 2, 4 and 8 days respectively. Untreated or vehicle-treated mice served as controls. Skin specimens were investigated by light, immunofluorescence and electron microscopy. Following pimecrolimus application hyperplasia of the epidermis and a dense dermal inflammatory infiltrate was observed. In contrast, these changes were not seen after tacrolimus application. Mast cells often in close vicinity to nerve fibers were found to be degranulated following 2 and 4 day tacrolimus application and only after 8 day pimecrolimus application. Immunofluorescence revealed a reduced staining with SP and CGRP in papillary nerve fibers after the application of pimecrolimus or tacrolimus. At the ultrastructural level, nerve fibers were found to be regular without signs of degeneration or inflammation in tacrolimus or pimecrolimus treated skin. These data indicate that topical application of calcineurin inhibitors is followed by the release of neuropeptides possibly leading to mast cell degranulation. However, the potency of tacrolimus to affect mast cells and nerve fibers appears to be more pronounced in comparison to pimecrolimus. Therefore, the release of mast cell mediators after topical application of calcineurin inhibitors leading to the release of neuropeptides in peripheral nerves may provide an explanation for the transient observation of burning and pruritus.

0913

Identification of a T cell epitope in the immunodominant NC16-A region of BP180 in bullous pemphigoid

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Autoreactive T cells are attributed a key role in the pathogenesis of bullous pempghigoid (BP) by triggering antibody (Ab)-formation directed against the hemidesmosomal adhesion molecule BP180 and induction of dermoepidermal loss of adhesion. Our study objective was to identify BP180 T cell epitopes. Using baculovirus-expressed BP180 proteins we investigated autoreactive T cell responses in patients with active BP (n=20). Autoreactive T helper cells preferentially recognized epitopes within the immunodominant NH2-terminal region of the extracellular domain (ECD) of BP180, but in about 50% of BP patients T cell epitopes were also located throughout the ECD of BP180. T cell lines were cloned by limiting dilution and tested against overlapping 17mer BP180-derived peptides. With this approach, we identified a T cell peptide within the NC16-A domain of the ECD of BP180 which is also immunodominant for auto-Ab. This stimulatory BP180-pepitide was recognized in association with HLA-DRB1*1301 by an autoreactive CD4+ T helper cell clone. Application of truncated peptides and alanine substituted altered peptide ligands abrogated the peptide response specifically and revealed a 12mer core peptide with essential peptide residues at positions 1, 4-7, 9-11. This autoreactive T cell clone exhibited a Th0- mixed cytokine pattern with high levels of IL-5, but also IL-4, IL-10, TNF-a and IFN-g upon specific stimulation. The identification of this BP180-specific peptide supports the functional relevance of T cell responses in the pathogenesis of BP and represents the basis for the development of immunomodulatory strategies in BP.

0914

Toll-like receptor 4 in Th2 responses induced in the skin and airway

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The factors governing initiation of Th2 responses to environmental allergens are not well defined. We have identified distinct requirements for Th2 activation at two sites of natural allergen exposure; Th2 responses induced by inhaled (i.n.) ovalbumin (OVA) are IL-4 dependent, while those generated by epicutaneous (e.c.) OVA rely on IL-13. Recently, we have shown that Th2 induction by i.n. OVA requires innate immune system signals, being impaired in TLR4 deficient (TLR4d) mice or in the absence of LPS. Given its presence on keratinocytes and mast cells, we asked if TLR4 was also required for Th2 induction in the skin. Wildtype (WT) and TLR4d BALB/C mice were exposed to e.c. OVA (100 µg) on day 0 and challenged 2 weeks later with i.n. OVA. Both WT and TLR4d mice had equally robust Th2 responses, with equivalent OVA-specific IgG1 (274±60 vs. 363±117 µg/ml, respectively), total IgE (1130±147 vs. 1500±348 ng/ml), and airway eosinophils following i.n. OVA challenge (6.7±0.6 vs. 9.5±2.2 $x10^5$). Thus, Th2 responses generated in the skin were TLR4 independent. Given that Th2 responses induced by e.c. OVA are uniquely dependent on IL-13, we hypothesized that IL-13 in the cutaneous environment was bypassing the need for TLR4. If so, addition of IL-13 to TLR4d mice might restore inhalational Th2 sensitization. TLR4d mice were exposed to i.n. OVA (100 µg) ± IL-13 (5 ng), or PBS alone, on days 0-2 and challenged 2 weeks later with i.n. OVA. TLR4d mice initially exposed to i.n. OVA alone had no increase in airway eosinophils compared to PBS controls. However, TLR4d mice that received i.n. OVA+IL-13 during priming, versus OVA alone, had a significant increase in airway eosinophils (34±7 vs. 0.04±0.02 $x10^5$; p=.01), as well as OVA-specific IgG1 (126±23 vs. 2.5±2.0 µg/ml, p=.007) and IgE (235±122 U/ml vs. undetectable, p=.003). Thus, addition of IL-13 restored inhalational Th2 sensitization in TLR4d mice. The data suggest that IL-13 in the cutaneous environment may allow Th2 sensitization in the absence of innate immune system signals.

0915

The role of mature and immature dendritic cells in the induction and elicitation of contact hypersensitivity

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Dendritic cells (DC) are not only potent antigen presenting cells for the development of immune responses but also play important roles in the induction of immune tolerance. A crucial factor which decides immunogenic or tolerogenic potentials of DC is their maturation or activation status. Although the role of DC in hapten induced contact hypersensitivity (CHS) responses has been well demonstrated, the effect of DC maturation in the induction and elicitation of the response remains to be identified. In the current study, mature and immature bone marrow derived DC were isolated and applied in vivo to address this issue. We found that hapten labeled mature DC, but not immature DC, could induce CHS responses. However, the immunization with hapten labeled immature DC did not tolerize mice; a normal CHS response occurred when the mice were re-sensitized epicutaneously with the same hapten. Further experiments exhibited that immunization of animals with mature DC activated hapten specific T cells in the draining lymph nodes while hapten presentation by immature DC resulted in a very low level of T cell activation. During the elicitation phase, intra-auricular application of hapten labeled mature or immature DC resulted in similar levels of CHS responses in pre-sensitized mice. In vitro stimulation of hapten primed T cells with hapten labeled DC demonstrated that mature and immature DC induced a similar level of IFN-γ production by CD8+ T cells. Our studies indicate that hapten presentation by immature DC is ignored by naïve T cells, which may represent a mechanism for unresponsiveness of the immune system to cutaneous antigens under steady state in which DC do not undergo maturation. However, immature DC can activate primed T cells and elicit inflammatory reactions during the elicitation phase of CHS responses. These data have implications for both the immune responses to cutaneous antigens and for trials employing DC for vaccination.

0916

Effect of tacrolimus and CD4+CD25+ regulatory T cells on T cell activation and proliferation

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Atopic dermatitis is characterised by a persistent T cell infiltrate in the dermis. A subset of CD4+ cells (CD4+CD25+) has been isolated in humans and has been shown to have the ability to suppress T cell proliferation in vitro. Topical tacrolimus has been shown to be an effective treatment for atopic dermatitis. This study aims to explore whether addition of tacrolimus and CD4+CD25+ cells to cultured CD4+CD25- cells can have additive or synergistic effects in their suppression, as seen when used alone. CD4+CD25- and CD4+CD25+ were isolated from blood of healthy volunteers by MACS bead separation. CD4+CD25- cells were stimulated to proliferate with anti CD3 and irradiated blood mononuclear cells. Tacrolimus was added in varying concentrations and CD4+CD25+ in varying numbers to CD4+CD25- cells in culture. Proliferation was measured by radioactive thymidine incorporation and CD69 expression as a marker of cell activation was measured by FACS analysis. Tacrolimus induced a dose dependent inhibition of CD4+CD25- proliferation, indicating that CD4+CD25+ cells are not needed for the suppressive effects of tacrolimus. Tacrolimus and CD4+CD25+ cells have an additive effect on the percentage inhibition of proliferation of CD4+CD25- cells 61% (tacrolimus alone) vs 82% (1:0.25 ratio CD25-:CD25+) vs 92% (tacrolimus and 1:0.25 ratio CD25-:CD25+). Both tacrolimus at the IC50 and CD4+CD25+ cells inhibit activation as seen by CD69 expression in stimulated CD25- T cells (54.8% vs 30.8%). However when added together they do not appear to have an additive effect. The additive effect seen on inhibition of proliferation of CD4+CD25- cells with both tacrolimus and CD4+CD25+ cells suggest that they mediate their suppressive effects via inhibiting different stimulatory pathways.

0917

Mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide

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Mast cell (MC)-mediated activity against bacteria has surfaced as an important mechanism of innate immunity. Cathelicidins (Caths), a family of antimicrobial peptides with activity against bacteria, fungi, viruses and protozoa, are present in MCs of humans and mice. To test the function and regulation of Cath in MC, we challenged connective tissue-type MCs derived in vitro from mouse bone marrow with lipopolysaccharide (LPS), lipid A, and lipotechoic acid (LTA). In LPS-treated MC, the expression level of mouse Cath (CRAMP) mRNA determined by quantitative real-time PCR was 6-fold higher after 3 h than that found in controls. To verify that increased CRAMP mRNA would also result in expression of CRAMP protein, we challenged MCs with the same bacterial products and measured the amount of intracellular CRAMP by flow cytometry with an anti-CRAMP antibody. FACS showed an increase in CRAMP protein in MCs 48 hr after challenge with all bacterial extracts. Western blot analysis showed MCs contain the (CRAMP) localized in granules as the full length precursor form, but can process some to active peptide. To test the functional significance of these findings, the ability to directly kill Group A Streptococcus (GAS NZ131) by MCs from wild-type 129SvJ mice (MCswt) was compared to MCs derived from littermate CRAMP knockout mice (MCs-/-). MCs-/- showed a 50% reduction in the efficiency of bacterial clearance when compared to MCswt. Moreover, both wt and -/- MCs expressed mRNA for mouse beta-defensin 4 (mBD4). These findings, and the normal perivascular location of MCs in dermis, suggest the possibility that MCs may use multiple antimicrobial peptides to defend against systemic microbial invasion through the skin, and that Cath is a vital effector molecule in this process.

0918

Photopheresis inhibits subcutaneous T cell tumor growth via a cellular immuno-regulatory mechanism

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Extracorporeal photopheresis (ECP) is approved for the palliative treatment of cutaneous T cell lymphoma but its underlying mechanism of action remains unresolved. ECP involves the reinfusion of peripheral blood leukocytes exposed ex vivo to 8-methoxypsoralen (8MOP) and UVA light. It has been suggested that ECP induces differentiation of monocytes to dendritic cells due to physical perturbation. Subsequent phagocytosis of apoptotic T cells by dendritic cells and presentation of anti-idiotypic tumor cell determinants to CD8+ T cells mediates direct cytotoxic activity against the offending tumor cells. We present data suggesting that monocytes do not differentiate into dendritic cells following overnight incubation after ECP. We further demonstrate that all cells including lymphocytes, monocytes and dendritic cells die via apoptosis after ex vivo 8MOP/UVA treatment with no preferential death of activated T cells. Most importantly, 8MOP/UVA treated leukocytes alone (no tumor cells) inhibit subsequent tumor challenge. This is consistent with our findings that ECP treatment of humans and animals is effective even before the introduction of antigen (BMT and solid organ transplantation) suggesting promotion of a more generalized immuno-suppressive state that does not rely on the presence of pathogenic cells during treatment. We have further characterized this suppression and found it to be antigen specific and of a cellular nature as it is transferable to a naive animal (also see Maeda & Schwarz abstract). Our current hypothesis is that ECP generates tolerogenic antigen presenting cells, which induce antigen specific regulatory cells. We are currently investigating the nature of this antigen specific cell-mediated tolerance.

0919

B cells are not required as antigen presenting cells during the induction of low zone tolerance to contact allergens

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Hapten-specific low zone tolerance (LZT) induced by epicutaneous application of low doses of contact allergens is mediated by $CD8^+$ suppressor T cells inhibiting the development of contact hypersensitivity reactions. Several factors have been shown to affect the generation of peripheral tolerance but the responsible APC of tolerance reaction to epicutaneously applied contact allergens has not yet been identified. To evaluate the role of B cells as APC in the induction of low zone tolerance to contact allergens, we studied μMT mice, characterized by a nonsense mutation of the IgM heavy chain resulting in total deletion of B cells. Tolerizing doses of the contact allergen TNCB (0.45 or 4.5 μg per site, 5 times per mouse) were applied epicutaneously. Subsequently, for the assessment of tolerance induction the inhibition of the contact hypersensitivity reaction was measured by ear swelling. Performing this protocol resulted in robust LZT in WT and B cell deficient mice, indicating that B cells are not required for the induction of LZT. These data were confirmed by hapten-specific restimulation experiments: lymph node cells (LNC) or purified T cells from tolerized WT and B cell deficient mice showed markedly reduced antigen-specific proliferative responses, as compared to LNC or T cells derived from sensitized control animals. Analysis of Th1/Tc2 cytokines produced by LNC obtained from tolerized μMT mice showed a typical Tc2 cytokine pattern of LZT with high IL-4 and IL-10 and reduced IL-2 and IFN-γ production. Furthermore, adoptively transferred $CD8^+$ T cells from tolerized μMT mice induced a significant tolerance reaction in WT recipients, demonstrating the development of hapten-specific effector $CD8^+$ T cells of LZT in B cell deficient mice. These results demonstrate that B cells are not mandatory for the induction of low zone tolerance to contact allergens.

0920

IgE-facilitated allergen presentation in atopic dermatitis

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The pathogenic importance of allergen-specific IgE in atopic dermatitis (AD) is controversial. Previous reports showed that IgE bound to Fcε receptors on antigen presenting cells (APC), enhanced T cell activation by a process of facilitated allergen presentation (FAP). However methods were selected to maximise IgE-FAP, and included use of purified APC populations, allergen specific T cell clones or lines, haptenised allergen, and chimeric, hapten specific IgE. The clinical relevance of IgE-FAP has therefore been uncertain. We investigated the effects of medium supplemented with 10% fresh, IgE-containing autologous serum on the proliferation of peripheral blood mononuclear cells (PBMC) incubated with *Dermatophagoides pteronyssinus* (*Der p*) extract. PBMC from 13 AD patients responded in a dose-dependent manner to *Der p* with maximal stimulation indices (mSI) of 4.1-100.0, whereas normal donors did not respond (mSI <2.6, n=5). Heating of autologous serum at 56 C for 60 minutes, which irreversibly denatures IgE, caused major reduction of PBMC responses in 12/13 atopic samples (p=0.003, paired t test). Incubation of PBMC with blocking monoclonal antibodies 3G6 and MHM6 against the high and low affinity IgE receptors (FcεRI and FcεRII) respectively, produced inconsistent inhibition of PBMC responses to *Der p* in the presence of fresh autologous serum (n=6), probably because IgE bound to its high affinity receptor is not reproducibly displaced by blocking antibody. Flow cytometry confirmed that 3G6 blocking antibody cannot displace IgE from monocytes in short term (2 hour) cultures, and displacement of IgE by 3G6 in 3-day cultures could not be determined because FcεRI expression on monocytes diminishes with time. Finally, atopic serum was depleted of IgE by using rabbit anti-IgE-coupled Dynabeads. Autologous PBMC responses to *Der p* in cultures containing 10% IgE-depleted serum were reduced by 55-62% in comparison with cultures containing control serum (n=3). These experiments are the first that use serum IgE depletion to indicate the clinical relevance of IgE-FAP in atopy, and further validate IgE-FAP as a therapeutic target.

0921

Dysfunctional $CD4^+CD25^+$ regulatory T cells in psoriasis

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In murine models of autoimmunity, $CD4^+CD25^+$ regulatory T cells (Tr) are key elements in the maintenance of peripheral tolerance toward self-proteins. Because psoriasis is sustained by the ongoing activation of T cells responding to antigens in the skin, we asked if psoriatic T cell reactivity could be due to insufficient regulation by Tr. We found that total and skin homing (CLA^+) $CD4^+CD25^{high}$ Tr are present in equal proportions in normal and psoriatic PBMC. However, psoriatic Tr showed a markedly decreased capacity to suppress autologous $CD4^+CD25^-$ responder T cell (Tresp) proliferation following antigen specific stimulation (p=0.0001, n=9), as well as polyclonal TCR stimulation (p=0.015, n=3). To determine if this is due to Tr-resistant hyper-proliferative Tresp, we performed "criss-cross" experiments. Whereas normal Tr were fully capable of suppressing the proliferation of hyper-reactive psoriatic Tresp by > 78%, psoriatic Tr could only suppress normal Tresp by < 39% (p=0.016, n=2). These data clearly map the defect to psoriatic Tr. We next performed analytic and functional analyses of $CD4^+CD25^+CTLA\text{-}4^+$ Tr in psoriatic skin. By flow cytometry, the ratio of Tr:Tresp in psoriatic dermis and epidermis was calculated as 1:6 and 1:3, respectively. Using purified psoriatic dermal Tr, we found that, as in blood, the 1:6 ratio provided < 20% Tresp suppression, (whereas normal Tr suppress > 50% at 1:8). These data demonstrate for the first time that Tr are present in an inflamed target organ, and are functionally impaired in a human disease. The observations that the numbers and functionality of Tr in psoriatic skin are insufficient to restrain hyper-reactive psoriatic Tresp are also novel, and may explain chronic T cell activation in peripheral organs of inflammatory/autoimmune diseases such as psoriasis.

0922

Hyaluronic acid glycosaminoglycans are direct stimulants of endothelial cell activation

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Endothelial cells (ECs) respond to injury by release of a repertoire of cytokines and chemokines, and expression of cell surface adhesion molecules. Various endogenous proteins and pathogen components can activate this innate immune response. Additionally, glycosaminoglycans (GAGs) such as dermatan sulfate that are released by injury of the extracellular matrix may serve as signals to the EC of tissue damage. This study sought to determine how ECs respond to the unsulfated GAG hyaluronic acid (HA). Microarray analysis of primary cultures of human dermal microvascular EC identified 40 genes that are up-regulated at least 3-fold after 6h stimulation with purified 4-8 disaccharide HA fragments (sHA). The cytokine IL-8 was prominent among those responding, (44 fold increase). This increase in IL-8 mRNA was further confirmed by ELISA of protein in culture supernatants and shown to be time and concentration dependent with increases of 117-fold over a 72 hour period in response to 20 ug/ml sHA. IL-8 induction was abolished by digestion of HA with hyaluronidase, thus verifying dependence on HA and not contaminants undetectable by HPLC. To confirm these in vitro data, mice were injected IP with 100ug sHA and serum soluble ICAM-1 and MIP-2 (mouse IL-8 homolog) measured by ELISA. sHA induced MIP-2 and ICAM-1 in treated mice compared to the PBS control injections. These data illustrate a potential mechanism by which exogenous extracellular matrix components can stimulate EC expression of factors critical to the initial stages of the inflammatory response. Importantly, this can occur in the absence of microbial products. Recognition of GAGs may therefore occur through stimulation of similar pattern recognition events previously thought to be specific to microbial pattern recognition.

0923

Dendritic cells distinguish fungal yeast from hyphae by selective binding of dectin-2 to mannan-like carbohydrates on hyphae and by up-regulating IL-4 and IL-10 expression

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Having discovered that Langerhans and dendritic cells (DC) express the C-type lectin receptors, dectin-1 and dectin-2, and since dectin-1 was reported to recognize β-glucan on yeasts, we posited that dectin-2 may recognize carbohydrates on fungi. To identify such ligands using fluorescence and ^{125}I-Fc binding assays, we made a recombinant protein for each receptor (Dec1-Fc; Dec2-Fc) consisting of the extracellular domain and IgG Fc. Dec1-Fc bound the yeast forms of *Candida albicans*, whereas Dec2-Fc did not. By contrast, Dec2-Fc bound hyphal forms of *Candida* with 10-fold greater affinity than Dec1-Fc. Similar results were obtained using yeast vs hyphal forms of *Aspergillus* and dermatophytes (*M. audouinii, T. rubrum, E. floccosum*). Neither β-glucan nor Dec1-Fc disrupted binding of Dec2-Fc to hyphae, indicating that dectin-1/dectin-2 possess disparate ligands. By contrast, EDTA blocked binding of Dec2-Fc to hyphae (but not Dec1-Fc to yeast), indicating that receptor function of dectin-2 (but not dectin-1) is Ca^{++}-dependent. In similar assays, we screened carbohydrates for ability to block binding of Dec2-Fc to hyphae: mannan was most potent (100% block), followed by mannose and fucose (75%). Dec2-Fc itself bound to mannan-coupled agarose beads. Similar data resulted using dectin-2 gene-transfected COS-1 cells as binding substrates. Finally, we used RT-PCR to examine cytokine mRNA of mouse DC after culture with *Candida* yeast or hyphae. Constitutive expression of IL-4, IL-10, IL-12, and IFN-γ was not altered by yeast. By contrast, IL-4 and IL-10 were upregulated 5-10-fold by hyphae, with no change in IL-12 and INF-γ. We conclude that DC can distinguish fungal yeast from hyphae by selective binding of dectin-2 to mannan-like moieties on hyphae and by upregulating Th2 cytokine expression. Our findings support the concept that differential recognition of microbial components by APC may condition immunity generated against infection.

0924

Polymorphism of CTLA4 gene in the patients with pemphigus

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Pemphigus is an autoimmune, intraepidermal blistering disease. In addition to pathogenic autoantibodies, other mechanisms seem to be of importance in the disease development. Some reports suggesting that activated T cells may contribute to the pathogenesis of pemphigus have been published recently. The cytotoxic T-lymphocyte antigen 4 (CTLA4) is known to be an important modifier of T-cell activation with down-regulatory properties upon B7 engagement. The human CTLA4 gene contains an A/G dimorphism at position +49 in exon 1. It was proven that various autoimmune diseases are associated with this polymorphism in CTLA4, however, it has not been examined in pemphigus so far. The aim of our study was to analyze the association of the CTLA4 gene variation with pemphigus in a Polish population. Analysis of the CTLA4 gene polymorphism within exon 1 (A∅G transition, +49) was studied in 44 patients with pemphigus (27 with PV and 17 with PF, 25 women, 19 men) and in 223 unrelated healthy individuals. All studied subjects were of Polish origin and were also genotyped for HLA-DRB1, DQA1 and DQB1 genes. PCR, followed by RFLP (Ital), was applied to define respective alleles of the CTLA4 gene. Genotype GG was found to be more frequent in patients (20.4%) vs. controls (7.6%) (p=0.043; OR=2.68 with CI95%=1.12-6.41). The G-allele frequency was also slightly higher in pemphigus patients (42.0 vs. 33.4%). Moreover, the genetic association of the CTLA4 polymorphism with pemphigus seems to be independent of the HLA class II genes. Obtained results support the hypothesis about the complex inherited component in pemphigus development.

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CD4+ and/or CTLA-4+ T cells are key regulatory cells in peripheral tolerance

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We have previously reported that both single [K14-mOVA] transgenic (Tg) and double Tg [K14-mOVA X OT-I that have a T cell receptor that recognizes OVA peptide in association with MHC class I] mice express membrane-associated ovalbumin (mOVA) under the control of a K14 promoter. When injected with CD8+ TCR Tg T (Vα2Vβ5+) cells from OT-I mice, single Tg mice developed a graft vs. host disease (GvHD), whereas double Tg mice did not develop disease. These results suggest that, in double Tg mice, regulatory mechanisms prevent infused OT-I cells from inducing GvHD. To determine why OT-I cells fail to induce GvHD, we assessed the ability of various antibodies to reverse this peripheral tolerance. Double Tg mice treated with anti-CTLA-4 mAb or with anti-CD4 mAb developed GvHD after transfer of OT-I cells. However, anti-CD25 mAb or anti-CD8 mAb did not reverse this peripheral tolerance. We then analyzed the phenotype of Vα2Vβ5+ T cells in superficial lymph nodes (LN) of double Tg mice after OT-I cell transfer. Before injection, double Tg mice expressed 3 different CD3+ (Vα2Vβ5+) T cell populations in LN: CD3+CD8-CD4-(42%), CD3+CD8+CD4-(8%), and CD3+CD8-CD4+(50%). 7 days after injection of OT-I cells, Vα2Vβ5+ T cells were unchanged from baseline even when the mice were treated with the anti-CTLA-4 mAb, but the percentage of CD8 positive cells (75% of Vα2Vβ5+ T cells) was dramatically increased and they exhibited a $CD62L^{dim}$ (57%) activated phenotype. In contrast, in non-antibody treated double Tg mice, 80% of T cells exhibited a resting ($CD62L^{bright}$) phenotype. In double Tg mice, 4.9% of LN T cells were CD4+CD25+ T cells before injection. Although treatment with anti-CTLA-4 mAb induced GvHD in the double Tg mice, the population of CD4+CD25+ T cells did not change after injection. Thus, we could not identify a CD4+CD25+ regulatory T cell population in these mice. Together, our results indicate that, in double Tg mice, CD4+ and/or CTLA-4+ T cells prevent infused OT-I cells from inducing GvHD. Further elucidation of the mechanism(s) responsible for protection from GVHD in this model may provide new insights into peripheral tolerance.

0926

Vasoactive intestinal peptide stimulates nitric oxide production in psoriasis vulgaris fibroblasts-mast cell co-culture

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Nitric oxide (NO) seems to play some role in many physiological and pathological processes including psoriasis vulgaris (PV) lesions development. There are some reports demonstrating skin fibroblasts involvement in psoriatic processes triggering and maintenance. It is generally accepted that neuropeptides are important in the course of psoriasis. So, the aim of our study was to evaluate NO levels in fibroblasts cultures obtained from lesional skin of PV patients and healthy subjects co-cultured with human leukaemic mast cell line (HMC-1) - stimulated with different concentrations of vasoactive intestinal peptide (VIP - 10-6, 10-7, 10-8, 10-9 M). Fibroblasts from pasages 1-3 were used. NO levels were evaluated by Griess method after 72 h of culture in the medium devoid of phenol red and serum. It was observed that VIP inhibited NO production by PV fibroblast and HMC-1 monocultures in comparison to spontaneous NO production - at 10-9M of VIP NO production was mean 1.31 μM and 1.14 μM in comparison to 1.93 μM and 1.79 μM, respectively. No production was dose-dependent as for both monocultures and co-cultures. The higher the VIP concentration the bigger NO production in all cultures studied was observed. What is more VIP inhibited NO production in normal fibroblasts - HMC-1 co-cultures whereas stimulated NO production in PV fibroblasts - HMC-1 ones: VIP concentration (10-9 M)- 1.46 vs. 1.93 μM. our results point out at VIP importance in PV fibroblasts and mast cell interactions.

0927

The predisposing and protective effect of HLA-DRB1 alleles on pemphigus development in patients of Polish origin

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Pemphigus is an autoimmune blistering disease characterized by the presence of antidesmoglein autoantibodies. Both genetic and environmental factors probably play an important role in its etiopathogenesis. Various studies have shown that the HLA-DRB1*04 and -DRB1*14 alleles confer strong susceptibility to pemphigus in different ethnic groups. The genotyping for HLA class II has not been performed in Polish pemphigus patients so far. Our study comprised 44 patients (27 with pemphigus vulgaris, 17 - with pemphigus foliaceus), the control group included 100 healthy unrelated individuals of Polish origin. The genotyping of HLA-DRB1 alleles was done using the Dynal Reli SSO HLA-DRB test (Dynal, Norway). The chi-square statistic was applied to determine differences in alleles and genotype distribution between groups. P-values were corrected according to Bonferroni method for multiple comparison. All computational studies were done using SAS Genetics software. Alleles DRB1*04 and *14 were significantly increased in patients as compared to controls (29.5% vs. 1.0%, pcor<0.04 and 11.5% vs. 3.5% pcor=0.063; respectively). Moreover, the DRB1*04-DRB1*14 genotype occurred dramatically more frequent in pemphigus individuals (11.4% vs. 1.0%,pcor<0.05, OR(95%CI)=11.4(1.3-100.1)), which suggests synergic effect of both alleles. A significantly lower frequency of HLA-DRB1*15 genotype was observed in patients compared to controls (2.3% vs. 15.0%, respectively, pcor<0.05). However, since one of the studied patients carries the DRB1*04-DRB1*15 genotype, the predisposing effect of the DRB1*04 seems to override the protective role of DRB1*15. The results confirm literature data concerning susceptibility to pemphigus linked to the DRB1*04 and DRB1*14 alleles. However, some protective effect of the HLA-DRB1*15 allele needs further evaluation.

0928

Activation of the platelet-activating factor (PAF) receptor: crucial molecular step in psoralen+UVA-induced immune suppression

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PUVA has been used for many years as a very effective treatment modality for various skin diseases such as psoriasis and early stages of cutaneous T cell lymphoma. The mechanisms by which PUVA leads to clearance of skin lesions are not well understood. However, PUVA has immunosuppressive properties, which in turn may contribute to or even be totally responsible for its therapeutic effects. Work from our laboratory (Walterscheid et al, J Exp Med 195, 2002) has disclosed PAF, a potent phospholipid mediator, as one of the first molecular events for UV-induced immune suppression. In this study we used a specific PAF receptor antagonist and a selective COX-2 inhibitor to study the possible role of the PAF pathway in PUVA-induced systemic immune suppression in the model of delayed-type hypersensitivity (DTH) to Candida albicans. Six to 8 wk old C3H/HeNCr mice were painted on the their shaved backs with 8-methoxypsoralen (100 ug/100 ul ethanol) 30 min before exposure to 50 kJ/m2 UVA. At day 5, the mice were immunized by injecting 2 x 108 formalin-fixed Candida albicans into each flank, and at day 14 they were challenged by injecting 50 ul of Candida antigen into each hind footpad. The intraperitoneal injection of both the PAF receptor antagonist PCA-4248 or the selective COX-2 inhibitor SC-236 (0.2 ug/100 ul each; 15 min before UVA treatment) abrogated PUVA-induced immune suppression. These results indicate that the pathway of PUVA-induced immune suppression follows that of UV(B) radiation, in which PAF induction is followed by the production and release of prostaglandin E2 and immunosuppressive cytokines such as interleukin-10. A better understanding of the early molecular events after PUVA exposure may offer the opportunity to dissect the beneficial (i.e. therapeutic) from the detrimental (i.e. tumorigenic) effects of this treatment modality.

0929

The role of endothelin-1 on cutaneous mast cells: implications for tissue-remodeling and inflammation

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Endothelin-1 (ET-1) is the most potent and long-lasting vasoconstrictor produced by numerous cell types including endothelial and epithelial cells. Although ET-1 and mast cells have been reported to relate with vascular, inflammatory and fibrotic disease states, the effect of ET-1 on cutaneous mast cells has been unclear. This study aimed to determine whether ET-1 regulates functions of skin-associated mast cells. We utilized murine fetal skin-derived cultured mast cells (FSMC) for this purpose because of their strong resemblance to cutaneous mast cells. FSMC constitutively expressed ET-1 and its expression was further up-regulated by ionomycin or Toll-like receptor ligands, including LPS and peptidoglycan. Previously, we identified the expression of Toll-like receptor (TLR) 3 and 9 on FSMC but not murine bone marrow-derived cultured mast cells (BMMC) that represent immature mast cells. Interestingly, poly(I:C) (TLR3 ligand) and CpG DNA (TLR9 ligand) up-regulated ET-1 expression on FSMC but not BMMC. FSMC also expressed both ETA and ETB receptors. Whereas ET-1 did not cause degranulation of BMMC, ET-1 potently caused degranulation of FSMC in a dose-dependent manner (10-6 -10-8M), which was completely ihnhibited by BQ-123 (an ETA antagonist) but not by sarafotoxin 6c (an ETB antagonist). Thus ETA mediates degranulation of FSMC in response to ET-1. Furthermore, pretreatment with monomeric IgE up-regulated degranulation of FSMC in response to ET-1. On the other hand, pretreatment with low dose of ET-1 (10-9-10-10M) significantly down-regulated degranulation of FSMC induced by crosslinking of FceRI. This down-regulation was again inhibited by BQ-123. Finally, ET-1 remarkably induced expression of TGF-beta1 and VEGF by FSMC. Thus ET-1 controls functions of cutaneous mast cells in many aspects. Such association of ET-1 and mast cells may play important roles in various cutaneous inflammation and tissue-remodeling.

0930

Heat killed mycobacterium vaccination completely suppressed severe dermatitis in AD-model mouse converting Th2 into Th1 cytokine profile

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Atopic dermatitis (AD) is a chronic inflammatory disease based on T-helper cell2 (Th2) immune responses. The recent increase of the prevalence of AD has been suggested caused by a cleaner environment and fewer childhood mycobacterium infections. While topical and systemic immunosuppressive medicine improved AD clinically, the reconstitution of the balance between Th2 and Th1 is an ideal therapy for AD. Here we report significant effect of a heat killed BCG vaccine in AD model mouse (Caspase-1Tg). Caspase-1Tg express severe itching dermatitis with elevated plasma histamine, IgE, IL-18, cutaneous mastocytes counts and lesional IL-4, IL-5 and IL-10 mRNA expression. After weekly vaccination with 20 micrograms of heat killed BCG suspension for 10 weeks, all the cutaneous manifestations were completely suppressed. According to improvement of the cutaneous manifestation, levels of plasma histamine, IgE, and IL-18 were normalized. The lesional IL-4, IL-5 and IL-10 expression were suppressed, but IFN-g mRNA was augmented. No relapse of the cutaneous nor laboratory manifestations of AD was observed over 6 months following-up after discontinuance of this therapy. No particular side effects was observed in this study. Thus, the heat-killed BCG vaccination is a highly potential therapy for AD, performing an essential role in AD therapy with restoring the balance of Th1/Th2 cells.

0931

DCIR & DCAR: antigen presenting cells expressing lectin-type paired immunoreceptors

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Dendritic cells (DC) play a central role on antigen specific immune response. Therefore regulating DC activation has been a major interest as for induction of tumor immunity and inhibition of autoimmunity. DCIR (DC immunoreceptor), which we have isolated from murine mature DC, is a type II membrane protein having extracellular C-type lectin domain and cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motif). Furthermore, we have obtained two isoforms of a gene similar to DCIR, by RT-PCR from murine splenic mRNA. These isoforms were named as DCAR (DC immunoactivating receptor) α and β, and β isoform lacks the neck domain. The extracellular lectin domain of DCAR is highly homologous to that of DCIR (91% identity), but the short cytoplasmic to transmembrane portion is rather homologous to that of dectin-2. Genes of DCAR, DCIR and dectin-2 are closely located on murine chromosome 6 near the NK gene complex. By RT-PCR using specific primer pairs, expression of DCAR mRNA was observed high in lung and spleen and low in lymph node and skin, while DCIR high in both spleen and lymph node. DCAR expression reached maximum on day 8 during maturation of bone marrow-derived DC, while DCIR on day 10. FACS analyses demonstrated the expression of those receptors on MHC class II positive antigen-presenting cells (APC) but not on T cells. Experiments using chimeric receptor of cytoplasmic DCIR and extracellular Fc γ receptor IIB revealed the ITIM-dependent inhibitory function of DCIR. In contrast, cotransfection experiments indicated that surface DCAR expression was induced only in direct association with Fc receptor γ chain, and the experiments using chimeric receptor of DCAR and Fc γ receptor revealed the activatory function of DCAR which was dependent on ITAM (immunoreceptor tyrosine-based activatory motif) of γ chain. Thus, a pair of DCIR and DCAR can possibly regulate APC function.

0932

Epidermal cytokine mRNA expression profiles in allergic and irritant contact dermatitis

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Although pathogesis of allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) seem to be distinct, comparative studies of ACD and ICD suggest that both the histologic and clinical features of ACD and ICD are strikingly similar. Immunohistochemistry studies of the expression of accessory molecules such as ICAM-ł and B7-1 also do not show a consistent feature that will distinguish ACD from ICD. In this study, we studied epidermal cytokine mRNA expression profiles in ACD and ICD in an effort to distinguish these two contact dermatitis. ACD and ICD were induced in mice ear by dinitrofluorobenzene and benzalkonium chloride, respectively. Histologically, ACD and ICD have in common intra-and intercellular edema, edema of the papillary dermis, and dermal mixed inflammatory-cell infiltrates; however eosinophilic infiltrate is more prominent in ACD. We separated epidermis from dermis using 20mM EDTA solution, and performed reverse transcriptase-polymerase chain reaction and RNase protection assay to study cytokine mRNA expression. The expression of IL-1β, IL-2, IL-4, IL-10, and CC chemokines(eotaxin, TARC, RANTES, JE) were increased in ACD, while the expression of TNF-α, IFN- γ, IL-12p40, IL-18 and CXC chemokines (IP-10, KC, MIP-2) were increased in both ACD and ICD. IL-1β, IL-4, IL-10 and CC chemokines might be considered as biomarker to distinguish ACD and ICD. In conclusion, the cytokine expression profiles between ACD and ICD are different, and some cytokines have been considered as biomarkers to distinguish these two contact dermatitis. In addition, the difference of chemokine expression profiles in ACD and ICD support the explanation of difference of leukocyte infiltration in ACD and ICD.

0933

Regulation of toll-like receptor-2 expression by adapalene: implications for the treatment of inflammatory acne

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The Toll-like receptors (TLRs) are implicated in directing recognition of microbial pathogens by cells of the immune system such as monocytes, macrophages. In particular, TLR2 recognizes the cell wall components of Gram+ bacteria, whereas TLR-4 mediates the response to the lipopolysaccharides (LPS) of Gram- bacteria. Upon activation, these receptors can trigger the release of pro-inflammatory cytokines. Activation of macrophages TLR-2 by components of *P. acnes* might contribute to the development of inflammatory acne lesions. Natural and synthetic retinoids, such as adapalene, are currently being used for the treatment of acne and we asked the question whether these drugs could have an effect on the regulation of TLR-2 expression by monocytes. Here we show that adapalene inhibits the expression of TLR-2 without affecting that of TLR-4. This effect was also observed with retinoic acid. The action of adapalene was dose dependent with an IC50 of 10nM. An antagonist of the retinoic acid receptors (RARs) completely inhibited the effect of adapalene showing that its action on TLR-2 expression is mediated by its interaction with RARs. Adapalene had no effect on the expression of other myeloid markers such as CD14 and HLA-DR. In addition, stimulation of monocyte TLR-2 by *P. acnes* induced the production of IL-12 and TNF-α and this induction was abolished by adapalene. Inhibition of TLR-2 expression may provide a novel mechanism by which adapalene exerts its beneficial effect on the treatment of inflammatory acne lesions.

0934

Stress affects atopic dermatitis possibly involving neurogenic pathways

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Stress may exacerbate atopic dermatitis. Respective research has largely focused on activation of the hypothalamus-pituitary-axis. Alterations in the nervous system have also been reported. E.g. the neuropeptide Substance P (SP) has been detected with contradictory results in patients with atopic dermatitis. SP has been shown to induce interferon-γ (IFN-γ) expression in T-Lymphocytes which normally express a TH-2 like cytokine pattern in vitro. TH-2 predominance is characteristic in acute atopic dermatitis in vivo, while IFN-γ expression is associated with the chronification of atopic dermatitis. However, local interactions between the nervous and the immune systems, especially under perceived stress, has never been reported in the context of atopic dermatitis. Here we show for the first time, that 48h after intradermal provocation with ovalbumin to induce a TH-2 dominated dermatitis and additional sonic stress exposure over 24 h, dermal infiltration e.g. by CD4+ T-lymphocytes is increased in the back skin of C57BL/6 mice. At the same time the number of SP-immunoreactive nerve fibers is decreased in the acute lesion. Stress without intradermal provocation with ovalbumin does not produce an atopic dermatitis-like infiltrate and alters the cutaneous innervation patterns only mildly, while provocation alone produces a milder infiltrate and mild alteration of the cutaneous innervation pattern. Sonic stress also increased IFN-γ levels, as determined by flow cytometry of peripheral lymphocytes. Thus we conclude, that SP-immunoreactive nerve fibers disappear from acute atopic dermatitis-like skin lesions due to the release of SP, especially under perceived stress. This may lead to an increased infiltration by immunocytes, which in turn is associated with a switch in cytokine-expression normally seen in chronic atopic dermatitis and provides first evidence for stress-induced exacerbation and phenotype-switch of cutaneous inflammatory diseases such as atopic dermatitis by interaction of the nervous and immune system.

0935

Human keratinocytes express functional IL-10 receptor after prolonged treatment with IFN-γ

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IL-10 is a promising candidate for the treatment of Th1-mediated cutaneous disorders such as psoriasis and allergic contact dermatitis (ACD). IL-10 possesses established immunosuppressive activities on professional antigen presenting cells, but it is still unclear whether IL-10 has direct effects on epidermal keratinocytes. The aim of this study was to investigate the presence of functional IL-10 receptor (IL-10R) in human keratinocytes isolated from healthy individuals and psoriatic subjects. Keratinocytes of healthy skin did not stain for IL-10R whereas ACD and, at lower extent, psoriatic epidermis showed an evident IL-10R reactivity in both basal and suprabasal epidermal layers. Primary cultures of resting and IFN-γ-activated keratinocytes obtained from normal and psoriatic donors were assayed for the mRNA expression of IL-10R α and β chains, and IL-10 binding capacity. RT-PCR analysis revealed the constitutive presence of IL-10Rβ mRNA and the absence of IL-10Rα mRNA in both normal and psoriatic keratinocytes. Cultures exposed for prolonged time (24-36 h) to IFN-γ expressed the IL-10Rα mRNA, which was present at higher levels in healthy keratinocytes compared to psoriatic keratinocytes. In contrast, IL-10Rβ mRNA levels were not influenced by IFN-γ. A significant IL-10-binding activity was measured on IFN-γ-stimulated, but not on resting cells, with healthy keratinocytes showing a higher avidity for biotin-labeled IL-10 compared to psoriatic keratinocytes. To test whether IFN-γ-induced IL-10R complex on keratinocytes was functional, we analyzed the IL-10-promoted early gene products, suppressors of cytokine signaling (SOCS). IL-10 induced a strong and sustained SOCS3 and some SOCS1 mRNA expression in keratinocytes pre-treated with IFN-γ for 36 h, but not in unstimulated cells. In aggregate, our results demonstrate that keratinocyte responsiveness to IL-10 can be obtained only after prolonged treatment with IFN-γ, which is mandatory for expression the IL-10R complex.

0936

A probable role of secretary IgA antibody to *Candida* in patients with severe type of atopic dermatitis and high tiers of IgE antibdy to *Candida*

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Recently, we have already reported that an impaired immune system to Candida of normal intestinal flora may be related to the deterioration of atopic dermatitis (AD), because the severity of the AD has correlated with IgE-RAST titers to Candida and many cases of the refractory AD has been improved by oral antifungal agents. The mucosal immune system is supposed to defence the mucosal invasion of Candida in healthy individuals, while its impairment permits their invasion and induces an increase in production of IgE antibodies to Candida. Then, as an indicator of the mucosal immune responsiveness to Candida, we measured the saliva levels of the secretory IgA (S-IgA) antibody specific to Candida and compared them with the serum levels of IgE-RAST titers to Candida in patients with AD and normal individuals, and analyzed their relationships to the severity of AD. The values of total sIgA and the sIgA specific to Candida fluctuated by sampling times. These fluctuations are supposed to be attributed to the water content of each collected saliva. We calculated, therefore, the ratios of Candida-specific sIgA to total sIgA, in order to get the individual values with little fluctuation. The results showed that the individual values thus obtained had little fluctuations. Therefore, we employed this ratio as the sIgA value specific to Candida for individual patients. The results indicated that the saliva levels of S-IgA to Candida in the severe AD patients were maintained within the lower ranges, while those in the mild showed frequently high values. On the otherhand, the saliva levels of S-IgA to Malassezia of normal skin flora were very low in either mild AD patients or not. These results indicate that the mucosal immune system to Candida appears to normally function in the mild patients, but dose not in the severe, suggesting that the impairment of the mucosal immune system may be involved in deterioration of AD at least as one of the important etiologic factors.

0937

The analysis of immune regulation of antinuclear antibody production induced by the injections of gold thiomalate in H-2 congenic mice and beige mouse

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The system has established that in susceptible mouse strains, H-2s, injections of goldthiomalate induce high serum titers of antinuclear antibody(ANA).The ANA produced reacts with fibrillarin as well as ANA detected in some patients with systemic scleroderma and mercuric chloride-induced ANA which we demonstrated recently.This is so called a hapten-induced autoimmunity model, of which mechanism of ANA production is still obscure. First, we tested ANA titers of three different genetic background of mice with H-2s haplotype and eight H-2 congenic strains on B10 genetic background. The results showed that all strains bearing H-2s(AsEo) were high responders, whereas AsEs, AsEk, AbEo, AkEo, ArEo were low or nonresponders. Then we searched other strain with I-E molecule lacked, and discovered beige mouse(bg) as a high responder. The bg has same genetic background of C57BL/6(AbEo) but differs from it only in a mutation of beige locus. Therefore, this beige locus is suggested to immunoregulate ANA production in this case. These results indicate the possibility that lack of I-E molecule and mutation of beige gene may be involved in an increase of ANA production. Treatment of anti-IL-4 against A.SW(H-2s) partially inhibited the increase in total ANA, but anti-IFN-r made them promote a week earlier than untreated one, indicating that Th2 cells preferentially play a role in this system. The ANA production induced by AuTM in A.SW was higher under the conventional condition rather than clean, indicating that enviromental factors are also related with ANA production as well as genetic factors like haplotype of I-A allele, lack of I-E molecule and mutation of beige locus.

0938

From phenotype to genotype: Functional analysis and chromosomal mapping of a hyper IgE mouse mutant generated by genome wide mutagenesis

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Laboratory animals carrying genetic mutations are of great value to study the biological function of genes. The ENU-Mouse-Mutagenesis Project aims at a large scale systematic production of mouse mutants using the mutagen ethylnitrosourea (ENU). Using this approach, we have identified mouse mutants with increased plasma IgE levels. One of these hyper IgE mutants displays additional immunological abnormalities. Flowcytometric analysis of peripheral blood and spleen lymphocyte subpopulations revealed dramatically reduced T cell numbers, while B cell counts were normal. Thymocyte analysis of mutants indicate a block in late T cell differentiation where numbers of CD4, CD8 double positive cells appeared to be normal, while single positive cells where almost absent. Bone marrow analysis of B cell development revealed an incomplete block of early B cell development at the stage of pre-B cells. Chromosomal mapping revealed a link to a 2.1 cM region localized on chromosome 1. Differential mRNA expression profiling of selected tissues using genome wide cDNA array technology is employed to narrow down potential candidate genes. Selected candidate genes are currently sequenced to identify relevant mutations. In conclusion, we have identified a novel mouse mutant with a combined phenotype of hyper IgE, a late block in T cell differentiation and an early block in B cell development. Chromosomal mapping and sequencing of candidate or novels genes will allow us to establish new gene/function/phenotype relations and may lead to novel animal models for human disease.

0939

IL-23 production by cosecretion of endogenous p19 and transgenic p40 in K14/p40 transgenic mice. Evidence for enhanced cutaneous immunity

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The proinflammatory cytokine interleukin 12 (IL-12), a p35/40 heterodimer, is produced by resident cells in skin and has been implicated as a pathogenic factor in T cell-mediated skin diseases. To investigate the effect of local IL-12 inhibition in cutaneous immunity, we generated mice which constitutively produce the IL-12 antagonists p40 and p40/40, but not p35/40, in basal keratinocytes (KC). Surprisingly, these mice spontaneously developed an eczematous skin disease. To identify the proinflammatory activity of transgenic (TG) p40, we searched for the presence of a moiety distinct from p35 which forms a proinflammatory cytokine with TG p40. Here we show that TG KC endogenously express p19, which, by cosecretion with TG p40 results in IL-23 (p19/p40) production. Repeated injections of recombinant IL-23 in littermate (LM) mice resulted in an inflammatory skin disease similar to that of p40 TG mice, confirming the proinflammatory activity of IL-23. Furthermore, IL-23 secretion by p40 TG KC induced elevated numbers of Langerhans cells (LC) with a marked upregulation of costimulatory molecules indicative of advanced maturation of K14/p40 LC when compared to LM LC. At the functional level, freshly isolated K14/p40 LC greatly exceeded LC from LM animals in their capacity to stimulate allogeneic T cell proliferation. To assess whether IL-23 regulates cutaneous immune responses in vivo, we used an allogeneic skin transplantation model. Full thickness skin grafts from K14/p40 donors (H-2q), transplanted across a MHC class I and class II barrier onto BALB/c (H-2d) recipients, were rejected in a significantly accelerated fashion (mean survival time [MST]: 8.8 days) when compared to skin grafts from non-TG LM (H-2q) (MST: 10.7 days, $p<0.01$). Based on these results, we propose that IL-23 may be a major player in cutaneous immune responses.

0940

Antitumor immunity utilizing melanoma cells fused with dendritic cells derived from MHC class I & II knockout mice

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Antitumor effects of dendritic cells(DCs) fused to mouse B16 melanoma cells have been reported previously. MHC class I & II in addition to tumor associated antigen,co-stimulatory molecules are known to be involved in crosstalikng between tumor cells and DCs. To assess whether MHC play an important role in induction of antitumor immunity by DCs,DCs derived from MHC knocked out mice as well as wild type were fused to B16 .FACS analysis demonstrated that levels of surface markers and phagocytosis of both wild and MHC knock out DCs were not significant.Animals were immunized by wild type or knocked out DCs fused to B16 twice every week followed by challenging of B16 melanoma cells.Outgrowth of implanted B16 was not significant between wild type and knocked out DCs.These results suggest that the levels of MHC may not have any effects on fusion cells based antitumor immunotherapy.

0941

Densities, distribution and phenotypic expression of T cells in human fetal skin

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T cells, which are at the heart of most adaptive skin immune responses, are present in normal human skin, but their occourrence in human fetal skin is poorly understood. T cell and Langerhans cell (LC) populations were studied, in human fetal skin, using single or double immunohistochemical stainings on cryostat section. Forty samples derived from different body regions of 13 individuals, ranging from 18 to 30 weeks estimated gestational age, were examined. In all specimens but one, we did not find any epidermal T cell. By contrast, dermal CD3+ T cells were present in all samples, mainly distributed in the perivascular compartment, with a statistically equivalent density, namely, a mean of 8 cells/centimetre linear section. Double-stainings showed that such CD3+ T cells were predominantly CD4+/CD45RA+/CLA-. On the other hand, LC, as assessed by CD1a expression, were evenly distributed within the interfollicular epidermis at all gestional ages, with an average density of 25 cell/centimetre linear section. Analysis of T cells and LC in different body regions did not show topographic differences in number and composition. We suggest that lack of epidermal T cells, although the LC network is fully represented, might reflect the scarce opportunity of fetal LC to contact foreign antigens in utero. Furthermore, the predominance of CD4+/CD45RA+/CLA- T cells in the dermis may reflect a random recruitment from the circulation.

0942

Contact hypersensitivity was attenuated in T-bet knockout mice

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T-bet is a Th1-specific T-box transcription factor that controls the expression of IFN-γ in Th1 cells and NK cells. Mice engineered for deletion of T-bet have been shown to be have a Th2-dominant immune response and show a phenotype reminiscent of human asthma when stimulated with methacholine aerosol (Finotto et. al, Science 295: 336-338, 2002). We evaluated the contact hypersensitivity response to trimellitic anhydride (TMA), a hapten known to cause asthma-like responses when inhaled, in T-bet knockout mice on both Balb/c and C57BL/6 backgrounds as well as in wild-type control animals. Mice were sensitized by topical application on the shaved abdomen of 100 µl 50% TMA on day 0 and 50 µl of 25% TMA on day 7. Contact hypersensitivity was elicited on day 14 by application of 10 µl of 10% TMA to both sides of the right ear. Ear swelling was assessed with a digital micrometer at various time points and the ears were removed for cytokine gene expression and histological examination. Total RNA was obtained using a freeze-smash technique and cytokine expression was quantitated by real-time PCR. Ear swelling in response to TMA was reduced 37% and 38% in T-bet -/- mice in comparison with wild-type Balb/c and C57BL/6 mice, respectively. The degree of inflammatory cell infiltrate and the presence of microabcesses were also reduced in T-bet -/- ear skin compared with wild type controls. IL-4 mRNA was increased in TMA sensitized ear skin of both T-bet -/- and wild-type animals. These results suggest that Th1 cells partially mediate contact hypersensitivity to TMA, but that Th2 cells also contribute to this reaction.

0943

Dendritic cells in allergic contact dermatitis--the widening spectrum

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The nature of the pathogenetically relevant antigen-presenting cells in the effector phase of contact hypersensitivity (CHS) is still unknown. To address this issue, we examined, by immunofluorescence, biopsies from positive 72h epicutaneous patch tests (EPTs; n=10) and normal human skin (NHS; n=5) for the presence of different members of the dendritic cell (DC) family. CD1a was used as marker for Langerhans cells (LCs), CD1c for dermal dendritic cells (DDCs) and CD123/BDCA-2 for plasmacytoid DCs (pDCs). \HS contained $CD1a^+$ and $CD1c^+$ cells in both the epidermal (19.3/mm- and 4.3/mm basement membrane, respectively) and dermal (16.2/mm^2 and 28.6/mm^2, respectively) compartment. In EPTs the number of dermal $CD1a^+$ cells (47.5/mm^2) was clearly increased at the expense of their epidermal counterpart (12.6/mm basement membrane). We obtained similar results for $CD1c^+$ cells of EPT specimens (15.7 epidermal $CD1c^+$ cells/mm, and 100.8 dermal $CD1c^+$ cells/mm^2). In contrast to NHS, a substantial proportion of epidermal (28%) and dermal (38%) $CD1a^+$ cells in EPT lesions expressed the activation marker CD83, whereas the majority of the $CD1c^+$ cells in EPTs remained unactivated. These findings may speak for distinct maturational events of LC and DDC in ACD. We also detected a small number of the newly defined $CD123^+BDCA-2^+$ pDCs in the dermal, but not in the epidermal compartment of NHS (2.9/mm^2). Notably, these cells represented a substantial proportion of the entire dermal DC infiltrate in EPT lesions. They were numerically increased already in 6h EPTs and peaked in 72h reactions (1.6 epidermal cells/mm, and 50 dermal cells/mm^2). FACS analysis of EPT reactions revealed that approximately 90% of $CD123^+MHCII^+$ cells bear BDCA-2. In summary we find that, in contrast to NHS, EPT lesions not only contain increased numbers of LCs and DDCs, but also a significant population of pDCs. It remains to be determined whether the attraction of pDCs to the site of hapten-challenge represents a bystander phenomenon or, alternatively, contributes to the evolution or resolution of the ACD.

0944

Neonatal epidermal cells impair the maturation of adult Langerhans cells *in vitro*

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We and others have previously shown that the fetal and neonatal epidermis contains $ADPase^+$, $CD45^+$, MHC class $II^{+/-}$, $DEC\text{-}205^-$ dendritic leukocytes which are defective in their capacity to stimulate antigen-specific T cell proliferative responses. Cells with a Langerhans cell (LC) phenotype (MHC class II^+, $DEC\text{-}205^+$, $Langerin^+$) appear only 3-5 days after birth. In the present study we tested whether a different cytokine milieu in the neonatal epidermal microenvironment compared to adult epidermis may be responsible for the immature phenotype of resident leukocytes. We therefore comparatively assessed the cytokine pattern of neonatal and adult cultures by testing epidermal cell (EC) supernatants for the presence of keratinocyte-derived cytokines known to promote survival and maturation of LC. While similar amounts of GM-CSF were measured in both culture supernatants during the entire culture period of 72 hours, the levels of TNF-α in the supernatants of neonatal-EC cultures far exceeded (10) those secreted by EC from adult animals. In coculture experiments we tested whether this finding may have any consequences for the maturation capacity of LC. Indeed, results obtained revealed that the presence of neonatal EC not only hampered the upregulation of costimulatory molecules (e.g. CD40, CD86) on syngeneic and allogeneic adult LC but also the expression of DEC-205. In contrast, adult EC failed to influence the phenotype of neonatal dendritic epidermal leukocytes. These findings may have important implications for our understanding of mechanisms underlying neonatal tolerance.

0945

The expression of Toll-like receptors, MD-2 and CD14; in mouse keratinocytes

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Toll-like receptors (TLRs), MD-2 and CD14 have been known to play an important role in innate immune responses to various microbial derived agents. Recently, we have demonstrated that lipopolysaccharide (LPS) and lipoteichoic acid (LTA), major biologically active components derived from gram-negative and positive bacteria, respectively, can activate human keratinocytes by CD14 and TLR4 dependent mechanism. However, the expression and biological function of TLRs, MD-2 and CD14 in mouse keratinocytes remains unclear. In this study we examined the expression of TLR2/4, MD-2 and CD14 in primary mouse keratinocytes and in the murine keratinocyte cell line PAM212. Our results indicate that TLR2/4, MD-2 and CD14 mRNA was constitutively expressed in primary mouse keratinocytes and PAM212 cells. The differentiation state of the murine keratinocytes had a significant effect on TLR2/4 and CD14 expression. Under high calcium culture conditions for 1 to 6 days there was a time dependent increase in murine keratinocytes TLR2, TLR4 and CD14 mRNA with differentiation. The results of these studies indicate that murine keratinocytes express TLRs and support the potential of a murine model system in examining the role of keratinocytes in mediating innate immunological responses to cutaneous infections.

0946

Profound defects in dendritic cell function in Sezary syndrome: correction with CpGs and CD40 ligand

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Patients with Sezary syndrome (SS) exhibit profound defects in cell mediated immunity partially resulting from marked deficiencies in the numbers of peripheral blood dendritic cells (DCs) and in their capacity to make the immune augmenting cytokines interferon α (IFNα) and interleukin-12 (IL-12). In an effort to enhance DC function of SS patients, we investigated the effects of several immunostimulatory compounds including repeat CpG nucleotides, which activate DCs following binding to Toll like receptor (TLR) 9 and soluble recombinant CD40 ligand (CD40L) which activates DCs following ligation of CD40. Initial studies indicated that TLR 9 and CD40 expression on DCs of SS patients was normal. Culture of patient peripheral blood cells with of CpG 2216 resulted in significant activation of DCs characterized by enhanced expression of CD83 and production of IFNα comparable to normals. Production of IFNα correlated with enhanced natural killer cell function in vitro. Following priming with low concentrations of IFNγ, culture of cells with CD40L induced high levels of IL-12 production, while IFNγ alone induced no IL-12. Thus, CpGs and CD40L, by virtue of IFNα and IL-12 induction, appear capable of activating both major families of DCs within the blood of SS patients. Because IFNα and IL-12 each exhibit therapeutic efficacy for SS, these findings have profound implications for the enhancement of anti-tumor immunity among SS patients.

0947

Identification of gene expression profiles that segregate patients with distinct primary cutaneous large B cell lymphoma

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Primary cutaneous large B-cell lymphomas (PCLBCL) account for 20% to 25% of all primary cutaneous lymphomas. Most PCLBCL are primary cutaneous follicle center cell lymphomas (PCFCCL) that present with nodules and tumors on a restricted area on the head and trunk. These lymphomas are highly responsive to radiotherapy and have an excellent prognosis (5-year survival 95%). In contrast, the PCLBCL of the leg have a higher relapse rate and a more unfavorable prognosis (5-year survival 58%). Methods: Using Affymetrix oligo-arrays (U95Av2) we determined gene expression profiles in biopsies from PCLBCL of the leg (n=7) and PCFCCL (n=4) containing more than 80% tumor cells Results: On average 55% of the genes were expressed in all biopies. Using unsupervised hierarchical cluster analysis, PCLBCL of the leg (n=7) and PCFCCL (n=4) are classified in two distinct clusters. Within these separate clusters gene expression profiles are relatively homogeneous. More detailed analysis (supervised clustering) revealed increase of 67 genes and decrease of 84 genes in all PCLBCL of the leg compared to all PCFCCL. In patients with PCLBCL of the leg 38 genes are associated with a detrimental clinical outcome (13 increased and 25 decreased expression) including genes involved in cell adhesion and proliferation. Discussion The differences in gene expression between PCLBCL of the leg and PCFCCL support the EORTC classification of PCLBCL. In addition, differentially expressed genes can provide insight in lymphomagenesis in PCLBCL and serve as prognostic and diagnostic markers as well as therapeutic targets.

0948

***In vitro* induction of specific Th1 cells using MVA-hTyrosinase infected dendritic cells**

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Since the incidence of invasive malignant melanoma is still increasing new vaccination strategies for tumor prevention and therapy are needed. Recently we could show in a mouse model that tumor-specific IFN-γ-producing Th1 cells are highly effective in tumor prevention and therapy of established disseminated lymphoma. In humans the use of gen-transfected dendritic cells (DC) seems to be more effective than the use of peptide pulsed DC for priming naive T cells. Gen-transfected DC can stimulate T cells independently from HLA-restriction and limited epitope sequences. Tyrosinase is expressed on more than 90% of malignant melanomas, and is an immunogenic target in autoimmune disease. Therefore we used a modified vaccinia virus ankara encdoing the human tyrosinase gen (MVA-hTyr) for gen-transfer in DC. Monocyte derived DC can be infected with a efficiency of more than 60% and express tyrosinase. Surprisingly, MVA-hTyr infection of DC abolished their capacity to stimulate autologous T cells *in vitro* even in presence of superantigen and IL-2. Phenotype analysis showed a decline of costimulatory molecules and induction of apoptosis in DC. *In vivo* vaccination with MVA leads to a potent immune protection in mice and human. This can be explained by phagocytosis of apoptotic DC by other DC for effective presentation. Thus, coculture of infected DC with non-infected DC restores the stimulatory capacity of DC in superantigen triggered T-cell stimulation. Incorporation of apoptotic DC by other DC could be shown using MVA-GFP. Three rounds of stimulation of autologous $CD4^+$ T-cells by DC-DC-MVA-hTyr led to generation of MVA-hTyr specific $CD4^+$ T-cells as shown by proliferation and cytokine production. Importantly, this *in vitro* generated Th cells presented an IFN-γ producing Th1 phenotype.

0949

Analysis of host-melanoma interaction of transgenic mouse melanoma model

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The immune system is not efficient enough to control melanoma progression despite the fact that it can recognize melanocyte differentiation antigens expressed by melanoma cells. Several oncogenes and tumor suppressor genes are implicated in melanoma progression. Of particular, the Ras-signaling pathway and Cdkn2a encoding Ink4a/Arf play major roles in development of melanoma not only in humans but also in the mouse melanoma model as described by Chin et al.. In order to elucidate host-melanoma interactions, we analyzed this model. Following Chin's method we crossed Tyr-Hras transgenic mice with Ink4a/Arf knockout mice twice to generate the mice with melanocyte-specific H-ras^{G12V} expression on an ink4a/Arf-deficient background. The mice develop spontaneous cutaneous and ocular melanomas around several months of age. Cutaneous melanomas form fairly-circumscribed tumors in the upper dermis, extending through deep tissues and are associated with epidermal hyperplasia. RT-PCR analysis of the tumor reveals transcripts of melanocyte differentiation antigens including mTRP-1, mTRP-2 and mMART-1. Immunohistochemical analysis reveals mild to moderate CD3 T cells infiltrating mainly at the periphery of tumor mass with scattered cells among the tumor. Scattered B220 cells (B cells and NK cells) are also seen at the periphery of tumor. More detailed analysis of host-tumor interaction and tumor escape from immune recognition is under investigation.

0950

Corticosteroids down-regulate murine Langerhans cell function *in vitro* in contrast to pimecrolimus

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Topically applied CS as well as the novel ascomycin derivative pimecrolimus are highly effective in the treatment of atopic dermatitis. To determine their effects on the immune functions of resident skin leukocytes, we comparatively assessed their influence on LC-driven T cell responses. BALB/c mice were topically treated with ethanolic solutions of the test compounds at their clinically used concentrations [1% hydrocortisone (HC), 0.05% clobetasol propionate (CL), 1% pimecrolimus] which inhibit efficaciously CHS responses in mice. Controls were treated with ethanol (vehicle) alone. Forty eight hours after the last application, epidermal cell (EC) suspensions were prepared from auricular and tail skin and cultured. After 2 days, nonadherent EC were counted and tested for viability, phenotype and function. While the viability of EC prepared from pimecrolimus- and vehicle-treated skin areas was greater than 70%, the percentage of trypan-blue positive EC from HC- and CL-treated mice was 45 and 61%, respectively. FACS phenotyping revealed comparable levels of surface-bound MHC class II molecules on LC from all groups. In contrast, the expression of CD25, CD205 and costimulatory molecules was significantly higher on LC derived from pimecrolimus- and vehicle-treated mice than on those of CS-treated animals. In line with this, LC-enriched EC (adjusted to equal numbers of LC) from pimecrolimus-treated mice induced a vigorous allogeneic T cell response comparable to that evoked by LC-enriched EC from vehicle-treated mice. In contrast, LC-enriched EC from CS-treated skin areas were greatly hampered in this capacity. These results show that, in contrast to CS, pimecrolimus does not interfere with the maturation and immune function of LC and immune function of EC.

0951

The percentage and activation phenotype of circulating CLA+ T cells in acute psoriasis suggest sequestration of skin-homing T cells to tissue

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The CLA antigen distinguishes memory effector T cells with cutaneous tropism. CLA+ T cells are important in the T cell mediated immune response in different cutaneous diseases like atopic dermatitis, drug induced allergic reactions and herpes infection. The purpose of this study was to determine by flow cytometry the percentage and activation stage of CLA+ T cells (both CD3 and CD4) in the PBMCs of psoriasis patients in acute phase, chronic phase, or in healthy controls. Psoriatic patients in the acute phase of their disease presented significantly the lowest percentagesof circulating CLA+ T cells of the three groups. Patients with acute psoriasis also presented the highest percentages of HLA-DR expression within the CLA+CD3+ or CLA+CD4+ circulating pool. The levels of CD25+CLA+ T cells were also the highest in the patients with acute psoriasis. There were no significant differences regarding the percentages of cells expressing HLA-Dr or CD25 within the CLA negative T cell subset in the 3 groups studied. In addition, purified CLA+ T cells from PBMCs of acute phase psoriasis patients incorporated Brdu and proliferated to exogenous IL-2 in the absence of TCR stimulation, in contrast to the CLA negative subset. Our data suggest that there is a decrease in the percentage of circulating CLA+ T celsl in acute psoriasis possibly due to a sequestration of circulating skin-homing T cells. Also that some circulating CLA+ T cells from PBMCs of acute psoriasis patients have been activated in vivo and probably come from cutaneous lesions. The differences in the percentages and activation state of skin-homing T cells within different stages of psoriasis are compatible with an early involvement of those cells in the pathogenesis of psoriasis.

0952

Heat shock proteins-27 and -70 are present in the epidermis and are important for the induction of allergic contact hypersensitivity

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Heat shock proteins (HSPs) refer to a large family of molecular chaperones. Their presence in the skin and their role in the pathogenesis of skin disease have received little attention. The purpose of this study was to determine which HSPs were present in the skin and to examine their participation in allergic contact hypersensitivity. Initial studies employed a proteomic approach to separate skin proteins from C3H/HeN and BALB/c mice. On 2-D gel electrophoresis spots with a Mr of 27.0 and a pI of 6.1 and a Mr of 71.23 and pI of 5.07 were noted. Protein identification by MALDI-TOF mass spectroscopy demonstrated that these spots had the characteristics of HSP-27 and HSP-70. Immunohistochemical staining of mouse skin with antibodies to HSP-27 showed that it was expressed throughout the superficial portion of the epidermis. To assess the functional role of HSP-27 and HSP-70, blocking antibodies were applied to the shaved abdominal skin of C3H/HeN mice. Two hours later, the animals were contact sensitized to DNFB and ear challenged five days after that. Animals in which sensitization was attempted through HSP antibody treated skin developed a reduced contact hypersensitivity response compared to mice that were not pretreated with antibody and to mice treated with an isotype specific control antibody. HSP-27 had a more profound inhibitory effect (48%) than HSP-70 (33%). When both antibodies were applied at the same time an additive inhibitory effect was observed (75% inhibition). The findings indicate that HSPs are present in murine skin and are important contributors to the development of contact hypersensitivity reactions.

0953

Corticosteroids but not pimecrolimus affect the viability of murine L Langerhans cells *in vivo*

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Given the importance of dendritic cells for immune functions, we investigated the effect of pimecrolimus, a novel anti-inflammatory drug for the topical treatment of atopic dermatitis, on the integrity and survival of Langerhans` cells (LC) in comparison with corticosteroids (CS). BALB/c mice were treated twice on one day on both sides of the ears and the tail with ethanolic solutions of the test compounds at their clinically used concentrations [1% hydrocortisone (HC), 0.05% clobetasol propionate (CL), 1% pimecrolimus] which inhibit efficaciously contact hypersensitivity responses in mice. Controls were treated with ethanol (vehicle) alone. Twenty four – 72 h after the last application, we observed an upregulation of Fas expression, induction of caspase-3 activity and fragmented DNA in most LC from mice treated with CS as determined by flow cytometry of epidermal cell (EC) suspensions and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining of epidermal sheets. In contrast, no such changes were observed in LC of pimecrolimus- or vehicle-treated animals. When we tested whether the topical application of CS blocks the secretion of soluble factors which are required for LC survival and maturation, we found that CS-EC supernatants but not pimecrolimus-EC supernatants contained significantly lower amounts of GM-CSF, TNF-α, and IL-1α than EC supernatants from vehicle-treated mice. The addition of GM-CSF to EC suspensions prepared from CS-treated mice greatly improved LC viability. In conclusion, these data show that CS but not pimecrolimus induce apoptosis in LC. This most likely occurs via both a direct and indirect mechanism. This confirms that pimecrolimus has a more selective mode of action than CS supporting a higher safety of topical pimecrolimus in the treatment of inflammatory skin diseases.

0954

Serum complement activation and T cell responses to whole microbes

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Complement activation is implicated as a proinflammatory process in autoimmune and inflammatory diseases of the skin and other organs. However, during investigations into the role of commensal and pathogenic skin bacteria as immunologic targets in atopic dermatitis, we have unexpectedly shown that complement activation is associated with decreased T cell responses. Whole, formalin-killed, washed *Staphylococcus* (*S*) *aureus*, *S capitis* and *S epidermidis* were incubated for 5 days with autologous peripheral blood mononuclear cells (PBMC) in medium with 10% fresh, unmodified serum or serum that had been heated under complement-inactivating conditions. All samples caused substantial, dilution-related PBMC proliferation, but the maximum stimulation index (mSI) was significantly greater with heat-inactivated serum (123 ± 54) than with unheated serum (43 ± 22; means ± SD, n=18, p<0.0001, paired t test). Soluble subcellular fractions of the *S* species, prepared by lysostaphin digestion, sonication and centrifugation, also caused PBMC proliferation, but mSI values in the presence of 10% unheated and heat-inactivated serum were similar (54 ± 77 versus 57 ± 79 respectively; mean ± SD, n=42, p=0.25, paired t test). Because complement activation products were likely to be generated in unheated serum by whole bacteria via the lectin pathway, but not by soluble subcellular fractions, we asked whether complement fragments might inhibit T cell activation. Accordingly, PBMC proliferation in response to whole *S aureus* was shown to be considerably greater in medium with 10% C3-deficient serum (prepared by immunoaffinity depletion) than control serum (maximum counts per min 19,064 ± 2,600 versus 10,637 ± 3,164, means ± SD, n=4, p=0.006, paired t test). We propose that certain complement fragments downregulate T cell activation, and may serve to control post-infective autoimmune responses. Whether these effects are due to iC3b-dependent IL-10 production, C5b-9-mediated immunocyte apoptosis or other mechanisms, remains to be determined.

0955

Topical application of adenovirus-vectored anthrax vaccine

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Anthrax caused by Bacillus anthracis is usually a cutaneous disease in nature. However, based on the rapidly fatal hemorrhagic mediastinitis resulting from inhaling spores, the threat of intentional use of anthrax as a biological weapon has engendered great public panic. The two toxins secreted by B. anthracis are composed of binary combinations of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). The PA component binds to a specific cell surface receptor and causes internalization and translocation of LF and EF. The contemporarily licensed anthrax vaccine in the US was made from cell-free filtrate of a nonencapsulated attenuated strain of B. anthracis and is given as 6-dose subcutaneous administration within 18 months. The principal antigen responsible for inducing immunity is the PA, but there have been concerns over the safety and efficacy of protective antigen vaccine. By taking advantage of potent immunological induction property of skin, we have successfully developed a skin-targeted immunization system by topical application of a recombinant adenovirus which encodes foreign antigen. In the present study, recombinant adenovirus encoding PA (Ad-PA) or LF (Ad-LF) were constructed and were topically applied onto the skin of different mouse strains. The antibody response to PA and LF were examined by ELISA after topical immunization. High titers of antibody to PA and LF were observed in mice topically immunized with both Ad-PA and Ad-LF and were dose-dependent. In contrast, immunization with either Ad-PA or Ad-LF resulted in weak antibody responses. The efficacy of this novel anthrax vaccination approach will be examined by protection study. We hypothesize that vaccination against anthrax may be achieved in a simple, effective, economical, painless, and safe mode by inoculation of expression vectors encoding PA and LF in a needle-free manner. This approach potentially could immunize a large number of people against anthrax-based biological weapons in an emergency setting during a terrorist attack.

0956

Increasing the number of Langerhans cells in the dermis as an approach to augmenting the immunogenicity of cancer vaccines

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Cancer vaccines must be developed to target multiple antigens. Plasmid DNA vaccines, easily tailored to deliver multiple antigens, have been only moderately successful in their translation to human clinical use. One strategy to make plasmid DNA immunizations more effective may be by targeting the reservoir of professional APC in the skin, i.e. skin Langerhans cells (LC), and improving in vivo transduction of these cells. We evaluated whether local application of LC recruiting agents could result in increased numbers of LC in the epidermis, the cutaneous reservoir of LC, and in the dermis, where DNA vaccines would be administered. Application of cytokines such as GM-CSF and TNF were compared with the effects of imiquimod, a newer topical immunomodulator. Mouse ears were treated with intradermal GM-CSF, TNF, or topical imiquimod daily for three days, then ears were harvested at 24, 48, or 72 hours after the end of the applications (days 4, 5 and 6, respectively). IAd-immunolabeled LC in the dorsal ear epidermal sheets were counted and compared to IAd labeling in the underlying dorsal dermal sheets. The kinetics of epidermal LC recruitment and migration varied significantly between reagents. At baseline, epidermal sheets contained 862 LC/mm2. Imiquimod resulted in an initial fall in epidermal LC numbers to 564 LC/mm2 on day 4, then an increase to baseline by day 5. GM-CSF and TNF treated ears revealed decreased epidermal LC counts of 405 and 326 LC/mm2 by day 5. The kinetics of dermal inflammation was also different between groups. Dermal inflammation in the imiquimod group increased progressively over time, whereas inflammation in GM-CSF and TNF treated dermis initially peaked on day 4 and then subsequently fell. These results suggest that LC activators may have variable effects at increasing and maintaining LC in the dermis for in vivo transfection with DNA vaccines.

0957

Langerhans cell precursors are present in spleen, peripheral blood and thymus

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More than two decades have passed since Langerhans cells (LC) were shown to be bone marrow-derived. A recent study reported that epidermal LC are very long lived and, under normal conditions and without perturbations, either proliferate or are replenished from LC precursors in skin. The purpose of this study was to determine whether LC can also be derived from cellular sources other than bone marrow or skin. Cell suspensions from F1 ($H\text{-}2^{bxd}$) mice were prepared from peripheral blood, skin-draining lymph nodes (LN), mesenteric LN, spleen, thymus and bone marrow (positive control), and injected IV into sublethally X-irradiated (8.5Gy) parent ($H\text{-}2^{b}$) recipient mice. To accelerate LC engraftment by depleting host LC, we painted ears with 1.5% trinitrochlorobenzene (TNCB) at the time of cellular reconstitution. Donor-origin cells were detected with anti-MHC class II antibody (anti-$I\text{-}A^{d}$), by counting LC in epidermal sheets manually or by flow cytometry of spleens to determine the extent of chimerism at days 11, 14 and 28 after cell transfer. Splenocytes, peripheral blood cells, and thymocytes, but neither of the LN preparations, gave rise to epidermal LC. After splenocyte infusion, long-lasting and almost total chimerism (64, 58, and 94% at day 11, 14 and 28) was observed in recipient spleens and increasing LC engraftment was observed (0, 415 and 3036/ear sheet of donor-origin LC in non-TNCB treated ears and 577, 2584 and 3467 in TNCB-painted ears), indicating the existence of LC progenitors in the spleen. Chimerism was also seen in mice infused with bone marrow (97, 96, and 98% in spleen, and 983, 2178, and 9037 LC in non-TNCB treated ears and 10765, 10843, and 38782 LC in TNCB-painted ears). LC engraftment was more evanescent when peripheral blood cells were infused (1.3, 1.9, and 0.8% in spleen, and 0, 0, and 0 in non-TNCB treated ears and 23, 115, and 19 in TNCB-painted ears) or when thymocytes were infused (5.8, 2.4 and 2.7% in spleen, 0, 16, and 0 in non-TNCB painted ears and 66, 128, and 0 in TNCB-painted ears). Taken together, these studies demonstrate that LC precursors are present in spleen, peripheral blood and thymus.

0958

A single nucleotide polymorphism in the DEFB1 gene associated with antimicrobial peptide expression and oral *Candida* carriage

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Human β-defensin-1 (hBD1) is a constitutively expressed cationic antimicrobial peptide that may have a role in innate immune defense against the adherence and carriage of oral *Candida*. We previously identified multiple single nucleotide polymorphisms (SNPs) in the DEFB1 gene encoding hBD1 and showed that a SNP in the 5' untranslated region (UTR) of hBD1 is associated with protection from oral *Candida*. This SNP is a C to G change at position -44 relative to the ATG start site. The frequency of this SNP is 20% in the overall population, but 6% in diabetics with high *Candida* carriage and 58% in those with low *Candida* giving statistically significant odds ratios for a protective effect. Two closely flanking SNPs (-20 and -52) result in several haplotypes. Because the 5' UTR has been shown to modulate transcription, we tested the causal effect of the -44 SNP on gene expression. Chloramphenical acetyltransferase (CAT) reporter plasmids with the hBD1 5' UTR or control plasmids were constructed and transfected into COS cells. CAT expression was measured by a radioactive assay normalized to β-galactosidase expression. The experimental plasmid with the most common haplotype containing the -44 SNP (GGG) was compared to constructs containing the three most common haplotypes with a C allele at the -44 position (GCG, ACG, or GCA). The CAT reporter activity of the -44 SNP G allele was twice that of the identical haplotype with C at -44 ($p<0.01$), and about 30% higher than the other two haplotypes. The results suggest that the -44 SNP increases protein expression and may yield more hBD1 peptide, providing a protective effect against *Candida*. This could be critical in immunocompromised patients, in which innate immune responses may have an important role in preventing commensal organisms from becoming opportunistic pathogens. Studies on genetic polymorphisms in markers of innate immunity may enable innovative approaches to investigations of susceptibility to opportunistic infections.

0959

Effect on basal cell carcinoma tumor defense mechanisms after treatment with imiquimod 5% cream

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Superficial basal cell carcinoma (sBCC) tumor defense mechanisms were evaluated before and after treatment with imiquimod to assess changes in avoidance of tumor immune destruction. Six subjects with 1 biopsy-confirmed sBCC tumor applied imiquimod once daily 5 days/week until signs of initial tumor erosion; baseline and midregression biopsies were examined by immunohistochemistry (ICH) and quantitative polymerase chain reaction (PCR). Increased cellular infiltrate density was observed in all 6 subjects. B-cell lymphoma-2 protein (Bcl-2) staining of tumor cells decreased in 4 of 6 subjects after 2 to 5 days of imiquimod treatment; Bcl-2 staining was negative on tumor cells in 2 subjects. Intercellular adhesion molecule-1 (ICAM-1), interleukin (IL)-10, Fas-ligand (Fas-L), and transporter associated with antigen processing (TAP)-1 ICH staining tended to be unchanged or somewhat decreased following 2 to 14 days of treatment with imiquimod. On local endothelial cells, rapid cellular infiltrates associated with enhanced expression of ICAM-1 adhesion molecules were observed. Early lymphocyte responses were mixed cluster determinant (CD)4 and CD8 in nature. The CD4/CD8 messenger ribonucleic acid (mRNA) ratios decreased or remained unchanged in 5 of 6 subjects, and lower CD4/CD8 ratios tended to be associated with production of interferon gamma (IFN-γ). Cellular infiltrates were associated with increases in IL-10. An increased infiltrate density of CD68+ cells was noted in the midregression biopsies. Of the 18 adverse events reported during the study, 11 (61.1%) were coded by the investigator as mild in intensity and 7 (38.9%) were coded as moderate. The study findings suggest that topical treatment of sBCC with imiquimod may have a direct or indirect effect on tumor defense mechanisms, most notably Bcl-2 expression. Treatment with imiquimod was associated with local immune activation, including the cells of the adaptive immune system. Imiquimod, approved for treatment of external genital warts, is under investigation for treatment of BCC and actinic keratosis.

0960

Cytotoxic drugs improve the effectiveness of IVIg in pemphigus

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Intravenous immunoglobulin (IVIg) treatment of pemphigus vulgaris (PV) causes a rapid decrease in circulating pemphigus antibodies. This suggests that a rebound in the level of these antibodies, as occurs following their depletion in plasmapheresis, may limit the effectiveness of IVIg and that suppression of the rebound with cytotoxic drugs may improve the effectiveness of the procedure. To evaluate this possibility we examined the effects of IVIg on serum levels of intercellular (IC) antibodies and on clinical response in patients with active PV treated identically with IVIg given with or without an effective dose of cytotoxic drug. Twelve pts with active PV unresponsive to conventional therapy with systemic steroids were treated with a single cycle of IVIg (400 mg/kg/day for 5 days). Nine of the patients were concurrently given cyclophosphamide (n=8) or azathioprine (n= 1) at 50 mg/TID. Three patients were treated similarly with IVIg but given no (n=1) or inadequate doses (n=2) of these agents. Serum levels of IC antibodies decreased by an average of 73% from baseline level within 1 week of initiating IVIg in the 9 pts given a cytotoxic drug compared to 0% decline in the 3 pts given none or an inadequate dose. One of the patients treated without a cytotoxic drug, IC antibodies decreased by 50% within 1 week when IVig was repeated together with cytoxan. Two wks following initiation of IVIg, skin lesions were healed by approximately 80% in pts treated with a cytotoxic drug vs 17% in pts that received an inadequate dose. These results suggest that cytotoxic drugs improve the ability of IVIg to rapidly lower pemphigus antibodies and to control active PV unresponsive to conventional therapy.

0961

Application of human papillomavirus type 5 virus-like particles may partially overcome a specific immune defect in patients with epidermodysplasia verruciformis

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Recently it was shown that epidermodysplasia verruciformis (EV) is caused by nonsense mutations of two adjacent novel genes on chromosome 17qter (EVER1 and EVER2) (Ramoz et al. Nature Genet 2002). These genes are probably involved either in regulation of HPV-keratinocyte interaction or in the control of immune surveillance against EV-HPVs since EV patients do not respond to EV-HPV antigens. The aim of the study was to find out whether immunogenic booster by administration of specific HPV5L1 virus-like particles (VLPs) could stimulate local and systemic cell-mediated and humoral immunity in EV patients with confirmed mutations of EVER2. All 4 EV patients studied were found anergic to HPV5 VLPs as measured by T cell proliferation assay and IFNg production. The PBMC stimulated with HPV5 VLPs generated high amounts of IL-10, but normal amounts of IL-18. Highly purified HPV5L1 VLPs, with no detectable DNA contamination, were applied intradermally at increasing doses: 75mcg, 150mcg and 300mcg at 1, 2, 6 monthly intervals. Antibodies to HPV5L1 VLPs were evaluated by ELISA using the same antigen. A seroconversion was observed in two of four cases, both familial EV, one with widespread skin involvement and the other a 17-year-old girl with plane wart-like lesions since the age of 3, in whom the lesions partly regressed after the 5th VLP administration and entirely disappeared, amid a slight inflammatory reaction, after the 6th VLP application. In two remaining patients with widespread lesions and several carcinomas there was a slight increase in antibody titres and some flattening of wart-like changes. In one of them, after 5th VLP administration, local skin reaction of delayed type to VLPs became positive and IFNg production by VLP-stimulated PBMC increased. Our study suggests that stimulation with EV-HPV VLPs couls partially overcome the specific immune defect in EV patients.

0962

Tissue-specific tumor immunity is mediated by trafficking-related molecules fucosyl transferase VII and b7 integrin

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Inflammatory stimuli generate tissue-specific patterns of selectins, chemokines and cell adhesion molecules by which memory / effector T cells preferentially home back to the tissue in which they first encountered their cognate antigen (anatomic memory). Fucosyltransferase VII (FucTVII) regulates leukocyte homing via the generation of selectin ligands. Cutaneous lymphocyte antigen (CLA) is a FucTVII -dependent carbohydrate epitope that serves as an E -selectin ligand on human T cells. FucTVII appears to play a similar role in mice, although a murine CLA homolog has not been identified. In a similar fashion, b7 integrin plays a key role in gut homing. Both a4b7 and a(iel)b7 integrins, which interact with MadCAM expressed on gut endothelium, mediate homing into gut-associated inflammatory sites. We hypothesized that tissue-specific homing is important in anti-tumor immunity. To test this hypothesis, we examined rejection of allogeneic tumors transplanted into the skin and gut of wild-type C57BL/6 mice, as well as C57BL/6 mice deficient in FucTVII or b7 integrin. Wild-type C57BL/6 mice are able to reject allogeneic J558L tumors transplanted into skin or gut. We found that FucTVII -/- mice showed delayed rejection of J558L tumors transplanted into skin but normal rejection of identical tumors transplanted into gut. b7 integrin -/- mice, in contrast, showed delayed rejection of J558L tumors transplanted into gut but normal rejection of identical tumors transplanted into skin. Transfer of C57BL/6 T cells expressing wild-type FucTVII ablated skin tumor rejection defects in FucTVII -/- mice. In summary, mice deficient in skin or gut trafficking-related adhesion molecules showed defective T cell mediated allogeneic tumor rejection in the corresponding tissues. From these results we conclude that tissue-specific homing of T cells is an important element of anti-tumor immunity.

0963

***In vivo* transfection of cis element "decoy" against signal transducers and activators of transcription 6 binding site ameliorates contact hypersensitivity**

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We previously demonstrated that Signal Transducers and Activators of Transcription 6 (STAT6) plays a crucial role in the induction of contact hypersensitivity (CHS) (J Exp Med, 191; 995, 2000). Further, repeated elicitation of CHS has been reported to induce a shift in cutaneous cytokine milieu from Th1 to Th2. Therefore, we hypothesized that synthetic double-stranded DNA with high affinity for STAT6 could be introduced in vivo as decoy cis elements to bind the transcriptional factor and to block the induction of not only single elicitation of CHS (acute CHS) but also repeated (12 times) elicitation of CHS (chronic CHS), thus providing effective therapy for CHS. Treatment by transfection of STAT6 decoy oligodeoxynucleotides (ODN), but not scrambled decoy ODN, after the sensitization by application of 5% 2,4,6-trinitrochlorobenzene (TNCB), had a significant inhibitory effect on both the induction of acute CHS and chronic CHS. Histological analysis revealed that edema, infiltration of eosinophils, and degranulation of mast cells were all significantly reduced in STAT6 decoy ODN transfected mice at both acute CHS and chronic CHS. To examine the mechanism of the in vivo effect of STAT6 decoy ODN, we assessed the cytokine level of challenged skins by ELISA. Expression of IL-4 was reduced by transfection of STAT6 Decoy but not scrambled Decoy. This is the first successful in vivo transfer of STAT6 decoy ODN to inhibit the induction of single or repeated elicitation of CHS providing a new therapeutic strategy for not only CHS but also atopic dermatitis.

0964

High efficient transgene expression after terminal differentiation of DNA transfected dendritic cells

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Dendritic cells (DC) play critical roles in the development and control of immunity. For evolving immune therapies ranging from vaccine development to tolerance induction, the use of in vitro generated DCs is an attractive strategy for immunomodulation. Efficient non-viral transfection of DCs would significantly contribute to these efforts by enabling genetic engineering of DC populations, but has not yet been achieved. In the present study, we analyzed the mechanisms involved in efficient transgene expression by murine DCs. Using biolistic delivery of plasmid DNA encoding reporter proteins such as firefly luciferase and EGFP under the control of the human CMV promoter, we demonstrate that transgene expression increases dramatically in DCs terminally differentiated through CD40, or DC-T cell interaction mediated differentiation. Under these conditions up to 75% of transfected DC expressed transgenic proteins and peptides. We demonstrate that induction of high transgene expression was due to signaling occurring during the interaction between transfected DC and CD4 T cells or through DC-DC homotypic aggregation. Signaling of DC through terminal differentiation resulted in the translocation of the nuclear factors -kBeta (NF-kB), activator of protein-1 (AP-1) and cAMP responsive element (CRE) to the cell nucleus of DC and enhanced gene expression was blocked by inhibition of these factors or by blocking adhesion molecules involved in DC-T cell interaction. Importantly terminal differentiation of DC favored the loading of transgenic antigenic peptides into MHC-class I molecules that resulted in induction of strong CTL response. Taken together, these studies demonstrate that highly efficient transgene expression by DCs can be achieved by combining an appropriate DNA delivery method with appropriate DC signaling stimulus able to translocate the transcription factors required by the transpromoter being employed into the nuclei of DCs.

0965

***All trans*-retinoic acid induces production of IL-8 in human keratinocytes via increased phosphorylation of IκBα in the NFκB pathway**

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All trans-retinoic acid has various biological effects on the skin. Several groups have reported that all trans-retinoic acid causes skin inflammation. This led us to hypothesize that all trans-retinoic acid induces the production of inflammatory chemokines or cytokines in human keratinocytes. First, we examined whether all trans-retinoic acid enhances mRNA expression of various chemokines in cultured normal human keratinocytes. We found that 10^{-9} to 10^{-5} M all trans-retinoic acid induced IL-8 mRNA expression in a dose-dependent manner. The optimum increase was 5.5-fold at 10^{-5} M compared to vehicle control after 24 h. The amount of IL-8 protein in culture medium was measured by ELISA. All trans-retinoic acid induced IL-8 protein production by human keratinocytes, 25 pg/ml at 10^{-6} M and 150 pg/ml at 10^{-5} M. IL-8 is reported to be produced by IL-1 via the NFκB pathway in human keratinocytes. Therefore, we examined the effect of all trans-retinoic acid on the NFκB pathway. Interestingly, 10^{-9} to 10^{-5} M all trans-retinoic acid increased the phosphorylation of IκBα in a dose-dependent manner, optimally 1.7-fold at 10^{-5} M, whereas all trans-retinoic acid had no effect on the amount of IκBα protein. IκBα binds to NFκB and prevents the translocation of NFκB to the nucleus until it is phosphorylated. An adenovirus vector expressing a mutant IκBα was used to block the NFκB pathway. This mutant IκBα cannot be phosphorylated and prevents translocation of NFκB to nucleus. The mutant IκBα almost completely inhibited IL-8 production induced by 10^{-6} M all trans-retinoic acid for 48 h. Combined, these data demonstrate that all trans-retinoic acid induces IL-8 production by increasing the phosphorylation of IκBα in the NFκB pathway.

0966

Toll-like receptor-2 up-regulates IL-4-induced eotaxin production through signal transducers and activators of transcription (STAT)-6 activation in dermal fibroblasts

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Innate immunity acts as first-line host defense to multi-cellular organisms. Many recent reports have revealed the new roles of toll like receptors (TLRs) in mammalian immune system including TLR-2 mediated activation of mast cells. It is wellknown that CCR3 chemokine, eotaxin plays a central role in the development of Th2 allergic diseases, including atopic dermatitis, asthma, and nasal allergy. To clarify the role of innate immunity in Th2 allergic diseases, we investigated whether TLRs could modulate IL-4 and/or TNF alpha -induced eotaxin production by fibroblasts derived from atopic dermatitis patients and TLR-/-mice. As results, eotaxin production and mRNA expression were significantly upregulated in atopic fibroblasts in contrast to normal fibroblasts after IL-4 stimulus. TLR2, 4 and 9 were identified by human fibroblasts at mRNA. Pretreatment of fibroblasts by TLR-2 neutralizing antibody (Ab) inhibited about 30% of eotaxin production and mRNA expression in both fibroblasts. Westhern blot showed that STAT-6 phpsphorylation was also down-regulated in TLR-2 Ab treated fibroblasts, whereas NF kappa B expression was unchanged. Luciferase assay demonstrated TLR-2 Ab treatment had no effect on NF kappa B activation in both fibroblasts stimulated with IL-4 and TNF alpha. To confirm the action of TLR-2 on IL-4 signaling, fibroblasts of TLR-2 -/- mice were stimulated with IL-4 and TNF alpha. TLR-2 -/- mice fibroblast showed a markedly lower STAT-6 expression than wild type after both stimuli. Taken together, our studies provide new evidences that TLR-2 expressed in dermal fibroblasts regulates eotaxin production via STAT-6 signaling. Theses finding could suggest that TLR-2 plays a novel role in Th2 allergic inflammation of the skin.

0967

Differential effects of immunosuppressive drugs on TARC/CCL17 production in PBMC of atopic dermatitis

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Thymus- and activation- regulated chemokine (TARC / CCL17) is a Th2 chemokine which chemoattacts CCR4+ memory T cells. Atopic dermatitis (AD) is an inflammatory skin disease characterized by the predominance of Th2 type cytokines such as IL-4 or IL-5 in peripheral blood. We previously showed high levels of serum TARC of patients with AD. However, little is known about the regulation of TARC production in peripheral blood mononuclear cells (PBMC) of AD. We first examined TARC production in PBMC of AD patients or healthy controls with or without stimulation by dust mite allergens extracts during 7 days in vitro. Cultured PBMCs in AD patients without stimulation of DME for 7 days spontaneously produced TARC protein (603.7pg/ml), which was significantly higher than that of healthy controls (24.4pg/ml). When PBMCs were stimulated with DME, cultured PBMC of AD showed strong enhancement of TARC production during 7 days up to 1576.1pg/ml, which was higher than that of healthy controls (159.8pg/ml). RT-PCR experiments revealed that the subtypes of TARC producing cells in PBMCs after the stimulation of DME were CD4+ T cells, CD8+ T cells and monocyte derived dendritic cells. We next examined the effects of various immunosuppressive drugs (Tacrolimus, Cyclosporine (CyA), Dexamethasone (Dex)) on TARC production by cultured PBMCs with DME stimulation. When PBMCs were co-cultured with DME and immunosuppressive drugs, TARC production of PBMC from AD patients were inhibited at protein levels in a dose dependent manner of these drugs. RT-PCR experiments showed that these immunosuppressive drugs inhibited TARC mRNA expression by PBMC. Gel shift analysis revealed NFkB activity was differentially inhibited by these immunosuppressive drugs. We suggest that TARC signaling could be the target for the future treatment of AD.

0968

Expression and regulation of CD1d in keratinocytes

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CD1d is a non-polimorphic antigen-presenting molecule that has been suggested to play a role in immunosurveillance. The aim of the present study was to examine the expression of the glycosylated and nonglycosylated forms of CD1d in keratinocytes and to establish if they have distinct roles in psoriasis. The expression of CD1d was examined in synchronised HaCaT keratinocytes by real-time PCR and Western blot using clone 51.1.3. We found that the expression of the glycosylated form was maximal in proliferating cells, while the nonglycosylated form was detectable especially in differentiated cells. Investigation of CD1d gene expression by real-time PCR in healthy skin, psoriasis and atopic dermatitis revealed that CD1d is overexpressed only in psoriasis. Immunohistochemical staining of skin sections was performed using clone NOR 3.2 and 51.1.3. The NOR 3.2 clone stained the differentiated, subcorneal keratinocytes in healthy skin and atopic dermatitis and the whole epidermis in psoriatic skin. The 51.1.3 clone showed a diffuse staining in all skin samples, and was much stronger in psoriatic skin. Since the high resistance to infections in psoriasis might be partly explained by increased CD1d expression, the effect of pathogens and proinflammatory cytokines was also studied. We found that staphylococcal peptidoglycan, Candida albicans, IL-8 and IFN-gamma strongly induced CD1d mRNA expression. Lipopolisaccharide and tuberculin had no effect and human herpes virus 1 down regulated the expression of CD1d gene in a dose dependent manner. These results showed that the two forms of CD1d are expressed in different stages of keratinocyte maturation and both are overexpressed in psoriasis. CD1d may have a role in host defense against certain pathogens and the high expression of the nonglycosylated form in psoriasis compared to atopic dermatitis may be responsible for less frequent skin infections.

0969

***In vivo* elimination of CD25+ regulatory T cells leads to tumor rejection of B16F10 melanoma when combined with interleukin-12 gene-transfer**

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CD4+CD25+ T cells are an important population that play a critical role in the maintenance of peripheral self-tolerance. Recently, it was shown that the elimination of these cells by *in vivo* administration of anti-CD25 mAb caused the regression of highly immunogenic tumors in syngeneic mice. In this study, we examined whether B16F10 melanoma cells regressed with the elimination of CD25+ regulatory T cells. We found the melanoma cells were not affected at all by *in vivo* anti-CD25 mAb administration alone but tumor rejection resulted in all mice when the administration was combined with IL-12 gene transfer to tumor cells. *In vivo* depletion of NK cells or CD8+T cells cancelled the tumor rejection. NK cell depletion allowed IL-12-transfected B16F10 melanoma (B16/IL12) to grow from an early stage and resulted in a more rapid tumor growth of B16/IL12 than that in mice without administration of anti-CD25 mAb. On the other hand, CD8+T cell depletion did not affect the tumor growth in the early phase but allowed B16/IL12 to grow in rather a late phase and resulted in almost the same degree of tumor growth as in mice without administration of anti-CD25 mAb. In a previous study, we showed that the elimination of CD4+T cells enhanced the antitumor effect of B16/IL12 and induced vitiligo-like coat color alteration. Therefore, we also examined the frequency of the change to a vitiligo-like coat color in mice showing tumor rejection caused by CD25+ T cell elimination to compare with the mechanism enhancing the antitumor effects by CD4+ T cell elimination. The elimination of CD25+T cells did not induce vitiligo-like coat color changes, though that of CD4+T cells induced the change in 60% of mice. These results indicate that *in vivo* depletion of CD25+ regulatory T cells is a potent useful adjuvant in immunotherapy of B16F10 melanoma when combined with IL-12 gene-transfer.

0970

Differentiation-regulated expression of TLRs in human epidermal keratinocytes

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Recent investigations have demonstrated that keratinocytes express toll-like receptors (TLRs) that might have an important role in host defence. The aim of our study was to investigate whether the proliferation and differentiation state of keratinocytes modulate the expressions of TLR2 and TLR4. The expressions of TLR2 and TLR4 were examined in cultured keratinocytes, HaCaT cells and in normal human epidermis using quantitative real time RT-PCR (Q-RT-PCR), western blot analysis and immunohistochemistry. As differentiation models, 1.2 mM Ca^{2+}-treated cultured keratinocytes and synchronised, contact inhibited HaCaT keratinocytes were used. In the normal human epidermis anti-human TLR2 antibodies stained the suprabasal layer, while anti-human TLR4 antibodies stained the whole epidermis. In order to examine whether differentiation could lead to the increased expression of TLR2 in the suprabasal keratinocytes, the expressions of TLR2 and TLR4 were compared in Ca^{2+}-treated and untreated keratinocytes both at mRNA and at protein levels. Q-RT-PCR analyses showed that Ca^{2+} upregulated the expression of both TLR2 and TLR4. Western blot analysis showed increased expression of TLR2 isoforms but not that of TLR4 in keratinocytes treated with Ca^{2+}. In an in vitro differentiation model, proliferating HaCaT keratinocytes were grown to confluency and the expressions of TLR2 and TLR4 were monitored at 24, 96 and 124 hours after reaching confluent state. During the 124 hours differentiation period, the expression of TLR4 increased while the expression of TLR2 remained unchanged. These results suggest that differentiation and proliferation state of keratinocytes modulate the expression of TLRs that might be involved in diseases characterised by disturbed KC proliferation and differentiation.

0971

Impaired function of STAT1 involves hyperproliferation of keratinocyte in psoriatic lesions despite the elevated presence of IFN-γ

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Psoriasis is an inflammatory skin disease characterized by hyperproliferation of keratinocytes. Although the exact pathogenesis of psoriasis remains to be elucidated, it is likely to be mediated by activated T cells, which primarily release IFN-γ. The keratinocytes in the psoriatic lesions are highly proliferating despite the presence of IFN-γ, a potent antiproliferative cytokine, suggesting that there may be a defect in the IFN-γ signaling pathway in psoriasis. In an attempt to investigate the mechanism by which psoriatic keratinocytes are less sensitive to the antiproliferative effect of IFN-γ, we examined the IFN-γ signaling pathway in both human keratinocyte derived HaCaT cells and the psoriatic keratinocytes. We detected in the IFN-γ treated HaCaT cells the elevated production of STAT1, which is a transcription factor responsive to IFN-γ, and phosphorylation of STAT1 at the tyrosine residue Y701. This in turn increased expression of IRF-1, IP-10, and MCP-1, which are crucial genes regulated by STAT1 in the IFN-γ signaling pathway, indicating that the IFN-γ signaling in the HaCaT cells occurs normally. In contrast, the phosphorylation of STAT1 at the tyrosine residue was undetectable in the psoriatic lesions, even though the STAT1 expression highly increased in the psoriatic lesions where elevation of the IFN-γ expression was confirmed. IFN-γ and STAT1 were hardly detectable in the non-lesions and the skin biopsies from healthy individuals. We examined if the absence of the phosphorylated STAT1 was associated with the SH-PTP2, which dephosphorylate the phosphorylated STAT1, but did not detect any significant difference in its expression level between lesions and non-lesions. We have further demonstrated that transcription of the IFN-γ/STAT1 responsive genes such as IRF-1, IP-10, and MCP-1 remained unchanging in the psoriatic lesions compared to those in non-lesions. These data suggest that impaired function of STAT1 to IFN-γ in the psoriatic lesions may give rise to the hyperproliferative feature of psoriasis.

0972

Large scale transcriptional analysis of mechanisms of contact hypersensitivity in rats

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The contact hypersensitivity (CH) in laboratory animals is a commonly used model of an immunologically mediated inflammatory skin disease. However, the underlying cellular events are not fully elucidated. We have, therefore, examined transcriptional alterations in rat skin during the elicitation phase of CH. Female Sprague Dawley rats, sensitized against 2,4-dinitrofluorobenzene (DNFB), were challenged with 0.5% DNFB on the flanks and the skin dissected 8 or 24 hrs after the challenge. The RNA from the challenged sites and from similar sites of sensitized animals without challenge (controls) was extracted and hybridized on GeneChip microarrays (Affymetrix RU34A). Genes which differed significantly ($p: < 0.001$) by > 40% in expression at 8 or 24 hrs from controls were analysed. Among the 414 genes retrieved, heat shock protein Hsp70, metallothionein, alpha 2u-microglobulin, caspase 6, Gadd153, p53, iNOS, ICAM-1, Gro-1 and MCP-1 (genes related to cellular stress, apoptosis, cellular migration or inflammation) were strongly expressed as early as at 8hrs. IL-4 receptor, histidine decarboxylase and mast cell protease 8 were upregulated at both time points and may have revealed mast cell activation. A similar expression profile exhibited the genes of IL-6, CD14, MRP8, MRP14, MIP1 beta and MIP2 indicating an early infiltration of macrophages. Expression of genes associated with homeostasis of dermal and epidermal structures were either induced (matrix metalloproteases MMP3 and MMP12) or reduced (collagen 1a, collagen 3, claudin 3, profilaggrin) 8 and 24 hrs after challenge. These differences may have been associated with histomorphological changes. In conclusion, this type of analysis proves to be a powerful tool to obtain a broad and detailed description of the diverse molecular cascades involved in contact hypersensitivity.

0973

The aberrant expression of toll-like receptor 9 in the peripheral blood mononuclear cells of patients with psoriasis vulgaris

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It is well known that the pathogenesis of psoriasis vulgaris is related to various factors, including genetic, and environmental elements. Such as bacterial infection occasionally exacerbating the clinical manifestation in severe psoriatic eruption. Toll-like receptors (TLRs) are proteins that stimulate proinflammatory cytokine gene transcription in response to various microbial ligands. The regulation of TLR2 and TLR4, TLR9 is known to be an important factor in the cell mediated immune responses to bacteria, but the participation of TLR9 has not been yet studied profoundly enough in psoriasis patients. In this study, we examined the expression level of TLR2 and TLR9 mRNA in peripheral blood mononuclear cell (PBMC) obtained from psoriatic patients and healthy controls. TLR9 mRNA was significantly increased in psoriatic patients, whereas TLR2 mRNA was not. Next, we investigate the expression level of TLR9 level in PBMC obtained from psoriatic patients and healthy control, using flow cytometric analysis. As results, we observed that the expression of TLR9 in the cell surface was very similar in both normal and psoriatic PBMC. In contrast, surprisingly, the expression of TLR9 in the PBMC from psoriasis patients was significantly increased in the cytoplasmic fraction compared to the normal controls. These aberrant distribution patterns of TLR9 in psoriatic patient PBMC might suggest that bacterial derived factors such as TLR9 ligand, CpG DNA play important role in induction of psoriasis.

0974

The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release

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The neurotransmitter 5-hydroxytryptamine (5-HT), commonly known as serotonin, is stored at peripheral sites in mast cells and released from this peripheral source upon IgE cross-linking. Here we investigated the expression of serotoninergic receptors (5-HTR), signal pathway coupling and biological activity of 5-HT of human dendritic cells (DC), showing that immature and mature DC expressed mRNA for different serotoninergic receptors. Thereby, the mRNA of 5-HTR1B, 5-HTR1E, 5-HTR2A, 5-HTR2B, two splicing variants of the 5-HTR3, 5-HTR4 and the 5-HTR7 receptor were detected. Immature DC preferentially expressed mRNA for the heptahelical receptors 5-HTR1 and 5-HTR2, while mature DC mostly expressed 5-HTR4 and 5-HTR7. The mRNA expression level of the ligand-gated cation channel 5-HTR3 did not significantly change during maturation. Isotype-selective receptor agonists allowed us to show that 5-HT stimulated 5-HTR3-dependent Ca2+ influx in immature and mature DC. Moreover we revealed that 5-HTR1 and 5-HTR2 receptor stimulation induced intracellular Ca2+ mobilization in immature, but not in mature DC. Ca2+ transients were mediated via Gi/o proteins activation. Activation of 5-HTR4 and 5-HTR7 induced cAMP elevation in mature DC. Functional studied indicated that 5-HTR regulate the proinflammatory activity of DC controlling the release of the cytokines interleukin (IL)-1β and IL-8. In summary, our study implicate that 5-HT stimulated in a maturation-dependent manner, different signal pathways in DC. These data would point for a role for 5-HT in regulating the immune response at peripheral sites.

0975

Imiquimod treatment of cutaneous T cell lymphoma

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The neoplastic cells of cutaneous T cell lymphomas (CTCL) express type 2 cytokines and are attacked by type 1 cytokine expressing reactive lymphocytes. Since imiquimod induces IFNa, IFNg and IL12 and down-regulates IL4 and IL5, we asked whether it would be effective in CTCL treatment. Ten patients suffering from MF stage IA-IIA were treated by application of 5% imiquimod ointment (one lesion of 20 cm2, 5 times per week for 12 weeks). Effects were assessed clinically and by evaluation of skin biopsies taken before and 4 weeks after treatment (treated and untreated lesion). Of the 7 patients completing the study, two developed a clinical response (1 CR, 1 PR). Two additional patients showed complete clearing of the treated lesion. Histological evaluation revealed an increase in CD8+ cells paired with a reduced number and a loss of epidermotropism of CD4+ cells in the responding lesions. Assessment of the lesional mRNA expression of IFNg and IL4 by real-time PCR revealed a strong reduction of both, IFNg and IL4 in treated and untreated lesion of the complete responder, thereby indicating complete clearing of the lesions. In treated lesions of local responders, an induction of IFNg mRNA paired with only slight reduction of IL4 was observed. This may correlate with failure to induce a sufficient anti-tumour response despite induction of type 1 cytokines. The increase of IL4, found in treated and untreated lesions of non-responders, may indicate ongoing proliferation of type 2 tumour cells. In summary, our data prove principle and efficiency of topical imiquimod therapy in CTCL. With regard to the low overall response despite satisfactory local effects in our patients, treatment should be extended to at least one lesion per skin region.

0976

Rho proteins regulate ICAM-1 (CD54) expression in Hs294T melanoma cells

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The expression of intercellular adhesion molecule 1 (ICAM-1, CD54), a molecule pivotal in many inflammatory and immune paracrine interactions, has been highly correlated with malignant melanoma progression. In the current study we investigated the involvement of Rho GTPases in ICAM-1 expression in Hs294T melanoma cells. Treatment of melanoma cells with Clostridium difficile toxin B covalently modified Rho GTPases in vivo by UDP-monoglucosylation and almost completely inhibited interferon-γ(IFNγ) induced ICAM-1 expression. Western blot and RT-PCR experiments revealed in Hs294T melanoma cells protein expression of RhoA, RhoB, RhoG, Rac1 and Cdc42 as well as mRNA expression of RhoC and TC10, respectively. Overexpression of constitutive activated forms of Rho proteins such as RhoGV12, Rac1V12 and Cdc42V12 increased significantly constitutive expression of ICAM-1, whereas INFγ{induced ICAM-1 expression was significantly inhibited in dominant negative RhoCN19, Rac1N17 and CdcN17 transfectants and potentiated in constitutively activated RhoCV14, RhoGV12 and TC10V26 transfectants. ICAM-1 protein cell surface expression in transfectants was paralleled by alterations in ICAM-1 mRNA steady state levels as detected by Northern blot and cell surface expression of ICAM-1 protein in transfectants could be blocked by actinomycin D. In summary, the present study suggests that RhoC, RhoG, Rac1, Cdc42, TC10 control expression of ICAM-1 in melanoma. These observations suggest that disturbances in Rho GTPases function are a critical event in melanoma progression.

0977

CCR10 expression by malignant melanoma cells: implications for tumor growth and metastasis

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Malignant Melanoma shows aggressive primary tumor growth and metastasizes into local draining lymph nodes, lung, liver, brain and bone marrow. In contrast to other malignancies, malignant melanoma also shows a high frequency of skin metastases. Recently, we showed that chemokine receptors such as CXCR4 are involved in the development of organ-specific metastasis to lymph nodes and lung. Furthermore, we identified the novel skin-associated chemokine CCL27(CTACK, ALP, ESkine) and its receptor CCR10(GPR-2) which specifically mediate the recruitment of lymphocytes into the skin. In the present study, we demonstrate that malignant melanoma cell lines as well as primary malignant melanoma tumors express high levels of CCR10 mRNA. Notably, skin metastases of malignant melanoma show significantly increased CCR10 mRNA expression compared to primary tumors suggesting the selection for CCR10-high expressing malignant clones. Immunohistochemical analyses confirmed CCR10 expression by MelanA-positive melanoma cells. In vitro, CCR10 signaling mediated migration and invasion of malignant melanoma cells and significantly induced their proliferation. In vivo, neutralization of mCCL27 resulted in delayed primary tumor growth of human melanoma cells in a SCID mouse model. Taken together, our findings suggest that CCR10 expression by malignant melanoma cells plays a role in primary tumor growth and the development of skin metastases.

0978

Inosine stimulates chemotaxis, Ca2+-transients and actin polymerization in immature dendritic cells via pertussis toxin-sensitive mechanism independent of adenosine receptors

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Inosine is an endogenous purine nucleoside which is formed by adenosine deaminidase during adenosine breakdown and is released into the extracellular space from the sympathic nervous system or metabolically stressed cells. Here, we studied the biological activity of inosine on human dendritic cells (DC) which are specialized antigen presenting cells characterized by their ability to migrate into target sites and secondary lymphoid organs in order to initiate an adaptive immune response. In immature DC inosine stimulated Ca2+ transients, actin polymerization and chemotaxis. During the maturation process DC lost their ability to respond towards inosine with these activities. Experiments with adenosine receptor antagonists and pertussis toxin as well as desensitization studies suggested that the activity of inosine is mediated by a G protein-coupled receptor pathway independent of adenosine receptors. In summary, our study implicates that inosine might be another member of the trafficking controlling system of immature DC and mediates its chemotactic activity by pertussis toxin-sensitive mechanism independent of adenosine receptors.

0979

Human keratinocyte regulation of CD1d cell surface expression during terminal differentiation is post-translational, and is not limited by its association with beta2 microglobulin

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CD1d is a major histocompatibility (MHC)-like molecule that serves as a recognition element for the presentation of glycolipids to NKT-cells. This molecule is expressed by human epidermal keratinocytes (KC) in normal human skin in the subcorneal layer of the epidermis, but there is no information about the regulation of expression of CD1d during KC terminal differentiation. Cultured human KC that were terminally differentiated (DIFF), expressed CD1d on their cell surface, whereas undifferentiated (UNDIFF), cultured KC expressed CD1d in the cytoplasm and not on the cell surface, as detected by confocal microscopy. This indicates that cultured KC recapitulate differentiation-associated regulation of CD1d expression in vitro. RT-PCR was utilized to study KC CD1d gene expression, and indicated equivalent mRNA steady-state levels in both cell types. As well, western blotting of DIFF and UNDIFF KC indicated equivalent expression of the 45kD mature form of CD1d by both cell types, despite increased expression of involucrin by DIFF KC. Because the 45kD form of CD1d is dependent on beta2 microglobulin (B2M) for membrane expression in other cell types, the association of this molecule with CD1d in DIFF and UNDIFF KC was studied by immunoprecipitation with monoclonal antibodies specific for CD1d or MHC class I, followed by immunoblotting with a B2M specific monoclonal antibody. 12kD B2M was equally associated with both CD1d and MHC class I in both DIFF and UNDIFF KC. These data indicate that during terminal differentiation, normal human KC regulate membrane expression of CD1d by a post-translational mechanism, which is not limited by its association with B2M, and suggests other factors, such as lipids, may be important in its cell surface transport.

0980

Withdrawn.

0981

Characterizing the inhibition of squamous cell carcinoma growth by αβ T cells

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We previously showed that intradermal injection of the squamous cell carcinoma (SCC) line, PDV, elicits markedly increased tumor incidence and decreased tumor latency in T cell receptor-β knockout (TCRβ-KO) mice, relative to wild-type (WT) C57BL/6 recipients. Adoptive transfer studies showed that both CD8+ and CD4+ cells were required for optimal tumor immunity. CD4-KO, interferon (IFN)γ-KO, and β2-microglobulin (β2m)-KO mice also showed increased tumor incidence and decreased tumor latency. Thus, this is a powerful model for the better characterization of αβ T cell-mediated inhibition of tumor growth. To investigate the influence of the immune system on the growth of PDV SCC tumors in vivo, an investigation was made of the gene expression patterns of tumors growing in immunocompetent vs immunocompromised hosts. By real-time qRT-PCR, all tumors growing in TCRβKO mice expressed much higher levels of Rae-1, a MHC-IB gene known to be up-regulated in SCC, and a target for cytolytic T and NK cells, relative to tumors growing in WT mice (5.2±0.8 vs 11.3±1.3; $p \leq 0.001$). To identify other genes that might be altered in their expression patterns in tumors according to the immunocompetence of the host, we performed cDNA microarray analysis. This identified several genes of which 5 were selected for further analysis on a broad panel of tumors harvested directly from growth in WT, TCRβ-KO or IFNγ-KO mice. Strikingly, 3 genes (osteopontin, semaphorin-IIIA, and syndecan-1) showed consistent up-regulated expression by semi-quantitative RT-PCR in tumors explanted from immunodeficient mice ($p \leq 0.01$ for each gene). While these findings do not assign the expression of the up-regulated genes to either the tumor cells or to the supporting stroma, they nonetheless demonstrate that the growth of SCC cells as a tumor in vivo is under immunological influence that is reflected in reproducible changes in the expression of particular genes. The expression of particular genes may act as a marker of the status of immunological pressure on a growing tumor.

0982

CpG oligonucleotides inhibit tumor growth in a human melanoma NOD/SCID xenotransplantation model

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For patients with advanced malignant melanoma, the 5 year survival rate with current treatment modalities is less than 5%. Melanoma is a prime example of a malignancy resistant to chemotherapy. Therefore new strategies for its treatment are greatly needed. One approach is to stimulate the immune system using CpG oligonucleotides into mounting a response against tumor cells. CpG oligonucleotides are distinct oligonucleotides containing non-methylated cytidine residues in cytidine-guanosine dinucleotides with particular flanking bases (CpG motifs) and are capable of strong immune stimulation by mimicking bacterial DNA. We evaluated the *in vivo* antitumoral effects of CpG oligonucleotides against human melanoma xenografts in NOD/SCID mice. CpG oligonucleotides administered in single peritumoral s.c. injections three times per week resulted in increased numbers of dendritic cells in draining lymph nodes, increased plasma levels of IL-12 and significantly inhibited the growth of established tumor xenografts by 60% ($p < 0.016$) as compared to the saline control. We also identified a significant increase of F4/80 + cells in the CpG oligo treated tumors by immunostaining. Our findings demonstrate the anti-tumor activity of oligonucleotides containing immune stimulatory CpG motifs in a xenotransplantation model with absent B-, T- and NK cell function. As NOD/SCID mice lack NK1.1 + cells, the increase of F4/80+ cells in the tumor tissue points toward macrophages as the possible effector cells involved in the growth inhibition of melanoma xenotransplants.

0983

Differential splicing of cTAGE genes leads to two tumor-specific antigens

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cTAGE-1 is a cutaneous T-cell lymphoma (CTCL)-specific tumor antigen which we have recently identified by SEREX (PNAS 98(2):629). The present study was aimed to identify related genes and to evaluate their value as tumor targets. RACE-PCR and DNA screening of a testicular cDNA phage library led to 5 new members of this gene family (cTAGE-2, -3, -4, -5A, and -5B), which could be ascribed to 4 different genes localized on chromosomes 7, 13, 14, and 18. Two of these genes were found as different splicing variants: cTAGE-1 and -2 (chromosome 18p11.2) and cTAGE-5A and -5B (chromosome 14). Sequences from public EST and mRNA databases could be ascribed as further splicing variants to cTAGE-1 (-1B and -1C) and cTAGE-5 (-5C and -5D). Expression analysis using gene-specific RT-PCR revealed cTAGE-1, -1B and cTAGE-5A expression was restricted to testis and tumor tissues, whereas the other cTAGE-members were found in few (2 to 8) other control tissues (27 tissues tested). Tumor-specific protein expression of cTAGE-5A, -C and -D was confirmed by Western blotting. Sero-reactivity against cTAGE-1, -4, -5A and -5B was found only in tumor-patients (CTCL and melanoma), while cTAGE-3 was also perceived in 1/10 control sera. The immunogenic epitope of cTAGE-1 was determined by using epitope mapping and sera of two CTCL patients. Moreover, cTAGE-1, -4, -5A and -5B could be detected in most types of tumor tissues and cell lines, but to variable frequencies. Particular cTAGE-members showed a widespread expression profile in specified tumor entities, e.g. cTAGE-1 in CTCL (11/24) and mamma carcinoma (10/23), cTAGE-5A in CTCL (8/18), in melanoma (5/25), and in colon carcinoma (13/39). We conclude, that cTAGE-1 and cTAGE-5 are new cancer testis antigens and that tumor-specific splicing of cTAGE genes may lead to further new candidate proteins for specific immunotherapy of CTCL and other malignancies.

0984

Cellular infiltrates in superficial basal cell carcinoma after treatment with imiquimod 5% cream suggest a cell-mediated immune response

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Early immune responses in superficial basal cell carcinoma (sBCC) tumors were examined to determine if cell-mediated immunity (CMI) is responsible for imiquimod-induced regression of sBCC. Sixteen subjects with a clinically-diagnosed sBCC tumor participated in an open-label, nonrandomized, mechanism of action study; 12 subjects applied imiquimod 5 days/week for either 1, 2, or 4 weeks and 4 subjects applied placebo cream 5 days/week for 2 weeks. After treatment, excised tumors were evaluated for confirmation of sBCC and were verified for tumor-negative margin; immunohistochemical analysis was used to evaluate immune cell type and number. Infiltrates were consistent among subjects with verified sBCC (n=8). Cluster determinant (CD) 4-positive (CD4+) T cells were present by week 1 and remained at comparable levels through week 4. CD8+ T cells infiltrated at a slower rate than CD4+ T cells, with the highest level measured at week 4. Dendritic cell counts increased with time. Macrophage counts were highest at week 1 and declined slightly at weeks 2 and 4. Fewer neutrophils and γδ T cells were present compared with CD4+ T cells, CD8+ T cells, and dendritic cells, but there was a general increasing trend in neutrophil and γδ T cell counts over time. Application site reactions were the most frequently reported adverse event with 8 (57.1%) of 14 subjects in the imiquimod group reporting at least 1 such event. The most commonly reported application site reactions were inflamed papules surrounding treatment site (n=3); redness at treatment site (n=3); and itching at treatment site (n=3). In conclusion, imiquimod-induced regression of clinically diagnosed sBCC is associated with infiltration of CD+ T cells and dendritic cells by week 1. The results of this pilot study indicate that imiquimod may facilitate tumor destruction by an immune-mediated mechanism similar to classic CMI in the skin. Imiquimod, approved for the treatment of external genital warts, is under investigation for the treatment of BCC and actinic keratosis.

0985

Expression and sero-reactivity of tumor-specific antigens in cutaneous T cell lymphoma

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Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of extra-nodal non-Hodgkin lymphomas with primary manifestation in the skin with poor treatment options in the advanced stages. As basis for future immune-therapeutic strategies we have investigated the possible expression of tumor-specific targets in CTCL focusing mainly on so-called cancer-germline genes. cDNAs derived from 20 CTCL tissues and 4 CTCL cell lines were tested with 15 gene- and 4 gene family-specific primers by RT-PCR and confirmative Northern blotting. The most frequently detected mRNAs were LAGE-1 (55% with only partial coexpression of the splicing variants), cTAGE-1 (35%), MAGE-A9 (27%), and the GAGE-3to7 group (35%). Furthermore, we could detect NY-ESO-1 (21%) and a MAGE-A subgroup (15%), while sub-specification of the latter proved absence of MAGE-A1, -A2, -A3, -A6, and -A12. SCP-1 was found in only one specimen and a several antigens could not been detected in any tumor tissue or cell line (MAGE-B, GAGE-1,2,8, and all four RAGE genes). 95% of all CTCL samples were positive for at least one of the frequent mRNAs in RT-PCR (LAGE-1, NY-ESO-1, cTAGE-1, MAGE-A9, and/or GAGE-3to7). Using a secondary SEREX approach we could detect sero-reactivity in sera of CTCL patients against recombinant cTAGE-1 (10/29), GAGE (3/19), MAGE-A1 (1/18), -A3 (1/18), -A6 (2/18), and -A9 (4/18) protein, but not against LAGE-1b, MAGE-A4b or MAGE-A12 protein (n=19). We conclude, that certain cancer-germline genes can be detected frequently in CTCL and are able to elicit a systemic immune response. These candidate genes might therefore be promising targets for immunotherapeutic interventions in CTCL.

0986

Intradermal genetic vaccination: role of bone marrow-derived antigen presenting cells

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We have recently shown that intradermal (i.d.) injection of pDNA coding for the autologous form of Pmel17 induces a specific T cell response capable of protecting DBA/2 mice against a challenge with a lethal dose of Pmel17-expressing melanoma cells. In the present study, we attempted to explore the mechanism(s) of this protective response. To this end, we used β-galactosidase (βgal) as a surrogate antigen. A single i.d. injection of a CMV-driven VR1012 vector engineered to express the lacz gene led to the activation of βgal-specific CD4+ and CD8+ T cells as well as antibodies. Excision of the VR1012 injection site at defined time points after vaccination (d1, 4, 6, 8, 11, 13) revealed that, for a specific immune response to occur, the i-site has to stay intact for 8 days. To test whether migratory antigen presenting cells (APC) or resident non-leukocytic cells are the critical sensitizing cell population, bone marrow chimeras were employed. F1 (C57BL/6 x BALB/c) mice that had been reconstituted with either BALB/c or F1 bone marrow were used 3 months after transplantation, i.e. at a time when >95% of migratory skin DC were of donor origin. Following i.d. application of 50mg VR1012lacz, BALB/c-reconstituted F1 were found to harbor T cells that recognized an H-2Ld-restricted peptide epitope but none that reacted against an H-2Kb epitope potentially presented by resident skin cells which express both H-2d and H-2b MHC molecules and recognized by the T cells that had been educated in an H-2d/b thymic microenvironment. Our results suggest that cross-presentation by bone marrow-derived APCs is critical for the induction of antigen-specific, protective T cell responses following intradermal DNA application.

0987

Caspase-1 recruitment domain protein expression in psoriasis

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The cysteine protease caspase-1 is responsible for cleavage of the inactive precursors of IL-1β and IL-18 into mature, pro-inflammatory cytokines. It is synthesised as a zymogen which, on receipt of an appropriate signal, undergoes oligomerisation and autoprocessing. A number of molecules have been described recently which bind caspase-1 and possess a caspase recruitment domain (CARD), through which association occurs. The CARD proteins RICK, ASC and IPAF induce cleavage of caspase-1 on binding, whereas ICEBERG, COP and CARD-8 antagonise this process. We have previously shown that a) human keratinocytes produce proIL-1b and proIL-18 constitutively, b) keratinocyte caspase-1 is normally present in an unprocessed form and c) keratinocytes synthesise all six CARD proteins *in vitro*. Given the potential importance of caspase-1 processing in the regulation of inflammation, we were interested to determine whether epidermal CARD proteins could be detected *in vivo* and whether their expression was altered in inflamed skin. Lesional and non-lesional shave biopsies from six untreated chronic plaque psoriasis patients were snap-frozen. Total RNA was prepared and semi-quantitative reverse transcription (RT) PCR was performed to determine CARD protein mRNA levels relative to a housekeeping transcript. Whilst both non-lesional and lesional epidermis expressed mRNA for the pro-cleavage CARD ASC, there was no significant difference in relative levels. Interestingly, despite abundant keratinocyte expression *in vitro*, RICK mRNA was absent from all samples tested. Analysis of anti-cleavage CARD molecules demonstrated no difference in levels of COP, very low expression of CARD-8 but a significant upregulation of ICEBERG mRNA expression in lesional psoriatic epidermis. These findings indicate a complex regulatory mechanism for caspase-1 activation in human keratinocytes *in vivo* involving at least 5 CARD proteins and suggest a net suppression of caspase-1 activation is maintained in lesional psoriatic epidermis, possibly as part of a counter-regulatory feedback mechanism.

0988

PAX3 as a new melanoma-associated tumor antigen: identification of HLA-A2 restricted T cell epitopes

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Melanoma is a prime candidate for antigen specific vaccination strategies. One reason for failure of vaccination might be the use of inappropriate targets on melanoma cells. PAX-3 is a developmentally regulated transcription factor with expression in 77% of cultured malignant melanoma cell lines and the corresponding tissues. Since PAX-3 expression is restricted to embryogenesis and certain tumors such as melanoma, it might be a suitable vaccine target. We used the technique of inverse immunology to design Pax3 peptides. According to the theoretical binding scores of this peptides to HLA-A*0201, three natural and two heteroclitic peptides were synthesised. From their ability to stabilise MHC class I molecules on the surface of T2 cells we could show that the practical and theoretical peptide binding is comparable. To screen the T cell repertoire of healthy donors we raised T cell lines against the five peptides in presence of IL2 and IL7. The presence of peptide specific T cells was detected by HLA-A*0201 tetramers folded around one of the heteroclitic peptides. Moreover, T2 cells loaded with four of the five peptides could be killed by these CTLs. CTLs of melanoma patients raised against the PAX3 peptides could also kill the autologous melanoma cells e.g. were able to recognize the naturally processed peptides. HLA-A*0201 transgenic H2 class I-deficient (HHD) mice were used to analyse the immunogenicity of the PAX3 peptides in vivo. Ex vivo spleen cells from HHD mice immunised with the strongest HLA-A*0201 binder peptide could kill T2 cells loaded with the vaccinated peptide. Thus, PAX3 derived peptides define a new group of tumor antigens and are potential new targets for antigen specific melanoma vaccination.

0989

Critical role of IL-10-dependent preventive T cells, a novel subclass of regulatory T cells, in tolerance to contact allergens

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The induction of hapten-specific low zone tolerance (LZT) by epicutaneous application of low doses of contact allergens is mediated by $CD8^+$ suppressor T cells (Tc2) that secrete immunosuppressive cytokines such as IL-4 and IL-10. Here, we used IL-10-deficient (IL-10-/-) mice and normal wild type (+/+) animals, to better characterize the role of IL-10 in the induction of LZT. Tolerizing doses of the contact allergen TNCB (0.45 or 4.5 μg per site, 5 times per mouse) were applied epicutaneously. Tolerance induction was assessed by measuring the inhibition of a contact hypersensitivity reaction (ear swelling). We found that the induction of low zone tolerance (LZT) is IL-10-dependent as IL-10-/- mice failed to develop LZT unless IL-10 was injected during the induction phase of LZT, whereas IL-10 reconstitution during the effector phase did not normalize LZT responses in these mice. IL-10 induced LZT by promoting LZT effector T cell development, as T cells from tolerized IL-10+/+ mice, but not IL-10-/- mice, adoptively transferred LZT to naive mice and prevented Tc1-skewing as well as clonal expansion of allergen-specific T cells. During the induction of LZT, $CD4^+$ T cells were the only lymph node or skin cells that produced relevant amounts of IL-10. Thus, $CD8^+$ LZT effectors are unique regulatory T cells, best classified as preventive T cells (Tprev), as they require IL-10 to develop, but prevent immune responses independent of IL-10. As Tprev-dependent LZT responses can be improved by IL-10 in normal mice, these T cells may prove to be useful targets of strategies aimed at avoiding the development of allergies and other harmful immune responses.

0990

Comparison of B7-DC expression with B7.1 and B7.2 as well as TNF family costimulatory molecules on Langerhans cell-like XS52 cells

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Dendritic cells (DCs) present antigens to T cells and initiate immune responses. T cell activation requires at least two signals: one delivered by the interaction between MHC-peptide complex on DCs and T cell receptor (TCR), and the other released by interaction of costimulatory molecules on DCs with their receptors on T cells. Costimulatory molecules consist of 2 superfamilies: the B7 superfamily (B7.1, B7.2, B7-H1, B7-H2, B7-3 and B7-DC) and the TNF superfamily (CD40, OX40L, CD27, Light and 4-1BBL). Langerhans cells (LCs) are the DCs residing in the epidermis. It has been demonstrated that resident LCs express little B7 molecules, however following LC migration and maturation B7.1 and B7.2 are significantly upregulated. In addition, we have recently demonstrated that the novel B7 family member B7-DC is expressed on migratory mature LCs as well. To further compare LC B7-DC expression with classic B7.1 and B7.2 as well as with TNF family costimulatory molecules, we analyzed B7.1, B7.2, B7-DC, CD40, OX40L and 4-1BBL expressions on LC-like XS52 cells. Since XS52 cells are early precursor immature DCs, LPS was utilized to induce their maturation via triggering Toll-like receptor 4 (TLR4). XS52 cells were cultured in RPMI medium containing LPS (1 μg/ml) or medium alone for 24 hours, and then immunolabeled with various monoclonal antibodies. FACS analysis showed that unstimulated XS52 cells expressed low to moderate levels of B7.1, B7.2, B7-DC, and 4-1BBL, but not CD40 and OX40L. After 24 h-LPS stimulation, B7.1, B7.2, B7-DC, and 4-1BBL levels were markedly increased. By contrast, CD40 and OX40L expressions were only minimally upregulated. Our observations suggest that in addition to the classic B7.1 and B7.2 molecules, B7-DC and 4-1BBL are two new maturation markers for murine LCs.

0991

Antigen-specific modulation of experimental bullous pemphigoid induced by active immunization

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Bullous pemphigoid (BP) is a subepidermal blistering skin disease associated with autoantibodies against the hemidesmosomal proteins, BP180 and BP230. Experimental autoimmune BP (EABP) in mice can be induced by active immunization with murine BP180 autoantigen (mBP180). We have showed that EABP is dependent on both T and B cells and is mediated by anti-mBP180 autoantibodies. In this investigation, we studied peripheral tolerance induction as a strategy to prevent EABP and the mechanisms underlying this effect. We found that subcutaneous administration of mBP180 antigen suppressed EABP in C57BL/6J mice (n=10 for testing and control group). Protection from BP development was associated with a significant reduction in mBP180-specific cellular and humoral responses. Tolerance treatments resulted in suppressed lymphocyte proliferation induced by mBP180 antigen and antigen-specific B cell expansion. The change of antigen-specific B cell population in control mice increased from 15% after the first immunization to 41% after the boosting immunization, while no significant change of antigen-specific B cell population in testing group oft mice was observed (18% to 22%). The protective effect induced by mBP180 antigen was mediated by reduced number of IL-4-producing T cells, suggesting that a shift from a Th2 to a Th1 response could be the underlying mechanism for the antigen-induced tolerance. ELISA assay revealed that circulating anti-mBP180 autoantibody levels were significantly lower in mice with tolerance treatment than in the control mice. Therefore, peripheral tolerance induced by mBP180 is effective in suppressing EABP and represents a potential antigen-specific immunotherapy for BP.

0992

Granulysin and cathelicidin have antimicrobial activity against *Propionibacterium acnes*

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Acne vulgaris is one of the most common dermatology diseases. Current treatments with antibiotics and retinoids have several disadvantages including severe side effects. In addition, the overuse of antibiotics has resulted in resistant strains of *P. acnes*. Therefore there is a need for development of more effective therapy for acne. Antimicrobial peptides are evolutionarily conserved proteins that are part of the innate immune system and have been found to be active against a variety of microorganisms. We sought to determine if these naturally occurring peptides are effective against *Propionibacterium acnes*. Antimicrobial peptides including granulysin, human cathelicidin peptide LL-37, and dermcidin were incubated with *P. acnes* and colony-forming units were determined. Granulysin killed *P. acnes* in a dose-dependent manner with 70% of bacteria killed at a concentration of 10μM and 98% of bacteria killed at a concentration of 15μM. LL-37 also killed *P. acnes* in a dose-dependent manner with 93% of bacteria killed at 4μM. In contrast, dermcidin, a recently identified antimicrobial peptide found in eccrine glands was found to be ineffective in killing *P. acnes*. In addition, smaller granulysin peptides were synthesized and tested against *P. acnes*. Peptides that contained a helix-loop-helix structure were effective against *P. acnes* while those peptides that did not contain this secondary structure were ineffective. Finally, a colorimetric MTT (tetrazolium) assay demonstrated that granulysin and cathelicidin are not toxic to human keratinocytes and peripheral blood mononuclear cells. Our data indicate that naturally occurring antimicrobial peptides such as granulysin and cathelicidin are effective in killing *P. acnes* and are not toxic to human cells and therefore may have therapeutic applications for the treatment of acne vulgaris.

0993

E-selectin and ICAM-1 co-localize in mid to upper dermal vessels in non-inflamed human skin: a means for cutaneous immunosurveillance

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The efficient recruitment of leukocytes to areas of trauma and infection underlies the success of the cutaneous immune system. Inflammation initiates a complex series of steps that draw leukocytes to tissue sites. Skin-homing leukocytes expressing CLA (cutaneous lymphocyte antigen) bind to E-selectin located on endothelium, inducing their tethering and rolling. Firm arrest of these leukocytes on the vessel wall, mediated by LFA-1 (lymphocyte function-associated antigen-1) and ICAM-1 (intercellular adhesion molecule-1)), is required for transmigration into the skin. While the multistep process of leukocyte-endothelial interactions has been well established in inflammatory models, its involvement in a skin-specific immunosurveillance system has been controversial. E-selectin and P-selectin are generally considered to be absent in non-inflamed skin and upregulated during inflammation. Several animal models, however, have shown spontaneous rolling of leukocytes in postcapillary venules in skin, which has been attributed to the constitutive presence of E- and P-selectin. To determine whether a similar process occurs in humans, we performed immunohistochemical stainings of E-selectin on non-inflamed human skin removed from cosmetic surgeries. These surgeries showed a small subset of mid to upper dermal vessels with non-contiguous E-selectin expression. Double immunofluorescence stainings of E-selectin and CD31 confirmed E-selectin staining in vessels and demonstrated a larger distribution of dermal vessels that stained only with CD31. Using confocal microscopy, we also demonstrated co-expression of E-selectin and ICAM-1 in these dermal vessels. Thus, the constitutive presence of E-selectin and ICAM-1, which should be necessary and sufficient to induce extravasation of circulating activated CLA+ T-cells, could provide the foundation of a cutaneous immunosurveillance system. Manipulating the expression of these molecules could enhance treatment of inflammatory and malignant skin diseases.

0994

The topical immune response modifier imiquimod induces Langerhans cell migration in human skin explants

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Imiquimod (R-837), an imidazoquinoline amine, is an immune response modifier that can induce the synthesis of IFN-α and other cytokines in a variety of cell types including monocytes, macrophages and dendritic cells (DCs). Imiquimod has been shown to have potent anti-viral and anti-tumor properties, and clinically this compound has been used for the treatment of external genital warts and perianal warts. Recently, it has been demonstrated that imiquimod activates immune cells via the Toll-like receptor 7 (TLR7)-MyD88-dependent signaling pathway. Studies have demonstrated that three members of the TLR family can trigger DC migration and maturation: TLR4 interaction with LPS, TLR3 interaction with dsRNA, and TLR9 interaction with CpG motifs. Previous studies demonstrated that imiquimod stimulates migration of murine Langerhans cells (LCs), which are DCs residing in the epidermis. In this study we examined the effects of imiquimod on human LCs using a skin explant ex-vivo culture system. We hypothesized that TLR7 plays a role in DC migration and maturation. Five percent imiquimod cream or vehicle cream as a control was applied on the epidermal surface of skin explants and cultured in medium at 37 C for 1-7 days. Epidermal sheets were then prepared and immunolabelled by anti-CD1a/FITC. No change in LC density was demonstrated at 24 h and 48 h, however, morphologically imiquimod-treated LCs appeared larger and brighter when compared with vehicle treated LCs. After a seven day-treatment with imiquimod, the density of epidermal LCs was significantly lower than that of the controls, suggesting that the LCs migrated from the explant. Our observations suggest that imiquimod induces TLR7 activation and may play a role in DC migration.

0995

Production of human T cells from bone marrow progenitor cells using a skin-derived thymic construct

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The development of T cells requires a period of specialized education in the thymus, an organ that largely undergoes involution during late adolescence. This requirement for thymic education has been a barrier to the laboratory production of T cells that can be given to immunocompromised patients to treat infection or malignancies. *In vitro* systems for T cell development have thus far required the use of either human or animal thymus tissue, preventing their use in adult human patients. Utilizing the natural similarities between skin and the thymus, we have developed a system in which cells from the skin are reprogrammed to act as a surrogate thymus, supporting the *in vitro* development of mature T cells from bone marrow precursor cells. Keratinocytes and fibroblasts from adult human skin were expanded separately in culture and then seeded onto a three-dimensional tantalum coated carbon framework. $AC133^+$ ($CD133^+$) human bone marrow precursors cells were then added and the resultant construct was cultured for 3 to 4 weeks. Cells produced by the construct were analyzed by flow cytometry and T cell recombination excision circles (TREC) analysis. Using this system, we have produced T cells that have surface markers consistent with mature, functional T cells. The cells were $CD3^{hi}$, either CD8 or CD4 single-positive, and a significant proportion were $CD45RA^+$, suggesting a naive phenotype. Input hematopoetic precursor cells were negative for CD3 and for TREC expression, while output cells were $CD3^+$ and positive for TREC expression, indicating that these cells are newly developed T cells. A comprehensive phenotypic and functional analysis of these cells is underway. The ultimate goal of this project is to develop a method by which newly generated T cells, either naive or with engineered T cell receptors, can be produced for the treatment of diseases of individual patients using autologous bone marrow and skin biopsies.

0996

Rapid generation of functional dendritic cells from monocyte precursors

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Current protocols for the generation of human dendritic cells from blood monocytes involve a variety of time-consuming physical manipulations as well as extended incubations with cytokine cocktails designed to both differentiate and mature monocyte precursors. Our group has previously reported that the extracorporeal treatment of peripheral blood in a closed cast acrylic flow system, as part of a therapy termed "photophoresis", could lead to the comparatively rapid conversion of blood monocytes to functional DC. Factors identified as critical to this conversion included both transient interaction of monocytes with the device's plastic surface under defined flow conditions and the presence of apoptotic tumor cells as phagocytic targets. Here we describe a modification of this system that appears to drive this rapid differentiation to a DC phenotype by utilizing a blood/device interphase of improved physical characteristics and controlled flow dynamics. We report that the appearance of DC-associated markers could be induced after an extremely short (30 min.) exposure of PBMC to the modified device followed by a brief overnight incubation at 37 degrees. As a measure of monocyte differentiation post-treatment, we monitored the loss of expression the monocyte associated marker CD14 as well increased expression of cell surface MHC Class II, CD36 and intracellular CD83, hallmarks of transition to a DC phenotype. We are presently testing the functional status of these reagents in phagocytosis and antigen presentation assays. These results indicate that functional DC can potentially be generated extracorporeally without the need for apoptotic stimuli and without the expense and time investment of current protocols. Study of dendritic cell differentiation utilizing this new blood/biomaterial interphase may result in both enhanced understanding of the physiologic determinants of DC biology and also suggest improved pragmatic methodologies for generating cellular reagents for DC-based therapies.

0997

Clonal γT cell DNA in the lymphoid infiltrate of lichen sclerosus

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Lichen sclerosus (LS) is chronic sclerosing lymphocyte-mediated skin disease with a strong autoimmune association. Well developed LS during high disease activity is characterized by a dense lymphocytic tissue infiltrate. Low density infiltrates can be observed in early and late atrophic disease stages. About 50% of LS of all stages have been described to contain T-cells with a monoclonally rearranged γ-chain of the T-cell receptor (mγTCR) as evidenced in conventional PCR analysis. For a better understanding of these observations we further investigated 10 vulvar and penile LS with mγTCR by a PCR-based fluorescent fragment GeneScan analysis (PCR-GSA) which combines the advantages of high sensitivity with accurate quantification and size determination of PCR products on high resolution polyacrylamide gels. Clonal T-cells as low as 1% within a low density inflammatory background can be identified with the γTCR-PCR-GSA. We further analyzed the the infiltrate immunohistochemically with antibodies to CD3, CD4, CD8, cytotoxic granula granzyme-B and TIA-1, and to γδT-cells. In all cases with a mγTCR in the conventional PCR analysis we identified a single monoallelic peak in the GeneScan analysis. The percentage of monoclonal γTCR DNA contained within the peak was calculated and ranged from 1.4% to 20.5% of total DNA analyzed. The size of the clonal DNA ranged from 52 to 68 base pairs (bp), with several cases of LS containing clonal γ TCR DNA of 62 bp. The immunohistochemical search for T-cells corresponding to the low percentage of clonal γTCR DNA identified γδ-T-cells together with granzyme-B and TIA-1 containing cytotoxic T-cells as most likely candidates. The low percentage of clonal γTCR DNA in the inflammatory infiltrate speaks against a systemic neoplastic disease, and further supports the theory of a local antigen-dependent proliferation of T-cells and an antigen-related immune process in LS. Asγδ-T-cells are involved in primary immune responses to (foreign) antigens / pathogens, the presented findings allow to speculate that the LS-associated antigen may be an infectious agent.

0998

Gene expression profiling of blood-derived dendritic cells following exposure to a contact allergen

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Langerhans cell activation and subsequent migration from the skin are critical steps in the induction of allergic contact dermatitis. Therefore, to gain a fuller understanding of these early events, changes in the gene expression profile of human peripheral blood-derived dendritic cells (DC) were examined following exposure to a contact allergen. DC were generated from the adherent cell fraction of peripheral blood mononuclear cells by culture in the presence of granulocyte/macrophage-colony stimulating factor and interleukin (IL)-4 for 7 days. The resulting immature DC were treated for 24 hours with either 1 mM or 5 mM dinitrobenzene sulfonic acid (DNBS), the water-soluble analog of the strong contact allergen dinitrochlorobenzene. Total RNA was obtained and changes in gene expression were analyzed using Affymetrix U95Av2 Genechips®. Pairs plots indicated good correlation between replicates for the treated and untreated cultures. In the principal component (PC) analysis, a treatment related effect was clearly observed for the first PC (the component representing the highest source of variance). Comparison of mean signal values from the replicate cultures revealed 185 genes that were significantly different ($p \leqq 0.001$)between DC treated with 1 mM DNBS versus untreated control DC and 1340 significant gene changes between 5 mM DNBS treated DC and untreated DC. Significantly up-regulated genes included cytokine/chemokine genes (IL-8, IL-1RA) and genes associated with T-cell co-stimulation (CD86), protein modification (pro-cathepsin L) and transcription (TGF induced factor). Significant down-regulation was observed in some chemokine (CCL4, CCL2) and immune function (FcεRα, lectin receptor) genes. Information gained by this gene expression profiling will be used in the development of endpoint measures that can serve as the basis of an *in vitro*method for identifying potential skin sensitizing chemicals.

0999

Innervation and expression of sensory neuropeptides in murine contact allergy

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The density of nerve fibers, using markers for protein gene product (PGP)9.5, growth associated protein (GAP)-43, as well as expression of sensory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), was determined in a murine model of contact allergy using immunohistochemistry and RIA. Sixteen female Balb/c mice were used. Eight mice were sensitized with oxazolone and 6 days later challenged on dorsal surface of the ears, control mice received the vehicle. One ear was taken after 24 hours and fixed in 4% formaldehyde, or 10% formalin with 0.2% picric acid, sectioned at 14 micrometer and processed for staining using a biotinylated fluorescence technique, while the other ear was frozen and processed for RIA. Image analysis or manually counting was used. PGP 9.5 immunoreactive nerve fibers in inflamed ears were 1.6+-0.7 mm/section compared to control ears at 1.3+-0.9 mm/section, in the epidermis, and 4.8+-1.2 mm/section and 4.5+-2.9 mm/section, in the dermis. GAP-43 positive fibers in the epidermis were increased ($p<0.01$), 2.7+-1.0 mm/section in inflamed ears, compared to 1.3+-0.8 mm/section in control ears, also the case for the dermal fibers, 9.7+-3.2 mm/section compared to 5.5+-2.5 mm/section. Total number of SP and CGRP nerve fibers tended to increase in the inflamed skin, at 23.9+-13.6/section and 56.0+-29.3/section, respectively, in contrast to 16.2+-4.9/section and 51.0+-21.4/section, respectively, in control skin. RIA demonstrated a tendency to a decrease in SP concentration, 2.6+-0.5 pmol/g in the eczematous compared to 3.0+-0.4 pmol/g in control ears. CGRP concentration was significantly lower ($p<0.05$) in the inflamed ears, 11.5+-3.8 pmol/g, compared to controls, 16.0+-2.1 pmol/g. Our results indicate that nervous system plays a role in contact allergy.

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Evidence for viral and autoimmune contributions to cutaneous T cell lymphoma

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Cutaneous T cell lymphomas (CTCL) are a group of clinical syndromes resulting from abnormal proliferation of skin-homing T cells. A viral etiology has long been suspected, and some studies suggest involvement of HTLV-1, but these are highly controversial. We here present new evidence for the involvement of HTLV-1 tax protein in CTCL and also demonstrate molecular mimicry between tax and human keratin. Our experiments to screen for CTCL tumor antigens using expression cloning show that hnRNP-A1 is a candidate antigen with reactivity in 32% of CTCL sera compared with 7% of healthy controls. Because hnRNP-A1 is known to be a pathogenic autoantigen in HTLV-1-associated myelopathy via direct cross-reactivity to HTLV-1 tax protein, we then hypothesized that patients reactive with hnRNP-A1 would also react with tax. Analysis of patient serum samples shows tax-reactive antibodies in 46% of patients and 15% of healthy controls with a strong correlation between hnRNP-A1-reactive and tax-reactive samples. In one serum sample containing relatively strong reactivity to both proteins, direct cross-reactivity is demonstrated using immunoprecipitated antibodies. Furthermore, examination of the T cell receptor CDR3 binding domains of patients with advanced CTCL and dominant peripheral blood clones reveals structural motifs which match those seen in tax-binding T cell clones from HTLV-1 infected patients with adult T cell lymphoma. An additional finding is that the antibodies which bind hnRNP-A1 and tax also bind human keratin 13, which we have previously demonstrated to be a skin autoantigen. In summary, these data suggest that either HTLV-1 or a related virus may play a role in CTCL and that there is molecular mimicry between tax and keratin which could be related to the skin tropism characteristic of CTCL.

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Development of dendritic cell therapies for cutaneous T cell lymphoma

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The aims of this study were to investigate whether Dendritic Cells (DCs) could be generated from peripheral blood monocyte precursors obtained from patients with Sezary Syndrome (SS), loaded with autologous tumor antigens, and induced to express potent antigen presenting cell function characteristic of activated or "matured" DCs, as a direct prelude to clinical trials. Peripheral blood monocytes isolated from SS patients were cultured for 5 days in the presence of GM-CSF and IL-4 and then loaded with autologous tumor antigens by either fusion or co-culture to create DC-tumor cell conjugate vaccines. DC-tumor cell conjugates were then matured or activated using a proinflammatory cytokine "maturation" cocktail. DCs generated from SS patients expressed phenotypic and functional properties consistent with potent antigen presentation and comparable to those of DCs generated from normal volunteers. This included expression of DR, CD86, CD80, CD83 and CCR7, and the exhibition of potent T-cell stimulatory function as measured in mixed lymphocyte reactions. DCs were efficiently loaded with tumor derived proteins as evaluated by confocal microscopy and quantitated by flow cytometry. Generally, co-culture DCs with autologous tumor cells resulted in approximately 40% of DCs internalizing significant quantities of tumor derived proteins. DC-tumor cell fusion efficiencies approached 70-90%. In comparison to DC-tumor cell co-cultures, some inhibition of DC maturation was observed in DC-tumor cell fusion vaccines for some phenotypic markers evaluated. Our results demonstrate that functional DCs can be generated from peripheral blood monocyte precursors of SS patients. These DCs can be efficiently loaded with autologous tumor antigens, and "matured" or activated in vitro. They suggest that the method of antigen-loading could influence subsequent DC function. Taken together, these studies demonstrate feasibility for the design of clinical trials to evaluate the efficacy of DC-tumor cell conjugate vaccines for the immunotherapy of Cutaneous T-Cell Lymphoma.

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Disperse blue 124 is a strong sensitizing material

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(Objective) In this study, we investigated skin sensitization potential of disperse blue 124 using a guinea pig maximization test. (Materials) [Animals] Female Hartley strain albino guinea pigs were introduced at 4 weeks of age. [Test materials] We selected refined disperse blue 124. (Methods) [Primary irritation study] The optimal concentrations for inductions and challenges were investigated preliminarily. We used 3 animals for each test substance. We conducted intracutaneous and 24 hours open testing of disperse blue 124 (5, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05% ac.). [Procedure]The procedure of the Magnusson & Kligman's guinea pig maximization test was followed for induction. Ten animals were included in the sensitization group and the control group. [Readings] Reactions were made at 24, 48, 72 hours after the challenge, according to the Draize's criteria. A summation of numerical scoring of more than 2 was judged as a positive reaction. (Results) [Primary irritation study] We judged 5, 2.5, 1.0, 0.5% ac. were strong necrosis reactions and 0.25, 0.1, 0.05% ac. were weak necrosis reactions in intracutaneous testing. We decided 0.25% ac. as intracutaneous sensitizing concentration. We judged 5, 2.5% ac. were 1 point of erythema and 1% ac. was negative in 24hours open testing. We decided 5% ac. as percutaneous sensitizing concentration and 1% ac. as challenge concentration. [The guinea pig maximization test] The positive reactions were observed in all animals with 1 and 0.5% ac., 5 out of 10 animals with 0.1% ac., 4 out of the 10 animals with 0.05 and 0.01% ac. and 0 out of 10 animals with 0.005% ac. in the sensitization group. All animals were negative in the control group. (Conclusion) We concluded disperse blue 124 was a strong sensitizing material.

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Selective inactivation of the interleukin-10 gene in T cells results in enhanced contact hypersensitivity reactions

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Interleukin-10 (IL-10) is a potent suppressor of T helper 1 responses. It is secreted by activated macrophages, but also by the epithelia lining inner and outer body surfaces as well as by T and B lymphocytes. In order to elucidate the function of T cell derived IL-10, we have generated mice with selective deficiency for IL-10 only in T cells by means of the Cre lox-recombination system. Unexpectedly, the T cell specific IL-10 mutants spontaneously developed inflammatory bowel disease. The mutants also show enhanced contact hypersensitivity reactions as compared to control animals, while responses to nonspecific irritation of the skin are not enhanced. Furthermore, the T cell specific IL-10 mutants succumb to infection with an avirulent strain of T. gondii which is not harmful for wildtype animals. This phenotype is very similar to that of conventional IL-10 k.o. mice which lack IL-10 in all cell types and is probably a result of unbalanced T helper 1 responses. These results show that T cell derived IL-10 serves important regulatory functions which cannot be substituted for by IL-10 released from other cell types. Future experiments will aim at defining T cell subsets carrying the IL-10 dependent regulatory function.

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Decreased clearance of apoptotic cells and enhanced cross-presentation in milk fat globule-E8 (MFG-E8) deficient mice

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Milk fat globule-associated protein MFG-E8 is produced in large amounts in lactating and involuting breast and by thioglycollate-elicited macrophages, and MFG-E8 mRNA is abundant in immature dendritic cells. Known to bind av integrins via its RGD-containing amino terminus and phosphatidyl serine via its carboxy terminus, the predicted involvement of MFG-E8 in ingestion of apoptotic cells by macrophages in vitro has recently been confirmed. We generated MFG-E8-deficient (MFG-E8-/-) mice to assess the role of MFG-E8 in clearance of apoptotic cells and in cross-presentation in vivo. MFG-E8-/- mice were indistinguishable from normal littermates and were fertile. Although no gross morphologic or routine histological abnormalities were detected, the number of TUNEL-positive thymocytes in resting and dexamethasone-stressed thymi of adult MFG-E8-/- mice exceeded that found in controls. In vitro studies suggested that resident, but not thioglycollate-elicited, peritoneal macrophages from MFG-E8-/- mice ingested apoptotic thymocytes less efficiently than control macrophages. In an assay if cross-presentation, immunization of female 129Sv/B6 MFG-E8-/- mice with beta-2 microglobulin-deficient male C57BL/6 splenocytes resulted in more vigorous immune responses than immunization of wild-type littermate control mice. We conclude that MFG-E8 plays a role in clearance of apoptotic cells by resident macrophages and that MFG-E8 is not essential for cross presentation.

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Regulation of CTLA-4 Expression in Human CD4 T cells

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In a search for genes that are differentially regulated in human naive, CD45RA, and memory, CD45RO, CD4 T cells, we have identified CTLA-4 as a gene that is preferentially upregulated in the CD45RO T cell subset. CTLA-4 is a member of the co-stimulatory molecule, which negatively regulates T cell activation. In contrast to CD28, which is expressed constituitively, the expression of CTLA-4 is highly regulated. To characterized CTLA-4 regulation, we analyze the expression of CTLA-4 in T cell subsets. In CD4 T cells, we show that CTLA-4 can be induced rapidly in 50% of the cells in response to T cell activators. This pattern suggests that CTLA-4 is not restricted to traditional regulatory T cells, nor CD8 T cells. Indeed, CTLA-4 can be induced to a higher level in CD4 T cells than in CD8 T cells. We confirm the differential regulation of CTLA-4 expression in CD45RA and CD45RO T cells by demonstrating that stimulation through the T cell receptor using anti-CD3 and anti-CD28 results in 30% of CD45RO T cells expressed CTLA-4, whereas less than 3% of CD45RA cells expressed CTLA-4. This is supported by mRNA analysis of CTLA-4 transcripts. To futher investigate the mechanism of CTLA-4 expression, 6kb of the human CTLA-4 promoter was cloned into reporter constructs to analyze the region necessary for transcriptional regulation. Successive 5'-promoter deletion constructs were created to determine the region necessary for transcription regulation. In transient lucuferase reporter assays in T cells, we find that the first 500 base pairs of the 5'-promoter contained regions that yielded the highest level of luciferase expression. This region is important for mediating induction by T cell activators such as phorbol esters. We are presently identifying the protein factor(s) that interact with this promoter region to up regulate CTLA-4 expression. Understanding the mechanism of transcriptional regulation of CTLA-4 will contribute to insight into mechanisms for differential gene regulation in CD45RA and CD45RO T cells.

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Separating acute from chronic delayed-type hypersensitivity reactions with radiolabeled RGD-peptides

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Chronic and recurrent inflammation can lead to tissue destruction and regenerative processes while acute episodes may resolve without scar formation. Angiogenesis plays a major role in organ-specific autoimmune diseases caused by delayed type hypersensitivity reactions (DTHR) and contact hypersensitivity reactions (CHSR). To better understand the mechanisms leading to chronic inflammation, we investigated the activation of αvβ3-integrin in acute- (A-CHSR, one episode of inflammation) and chronic contact hypersensitivity reactions (C-CHSR, recurrent episodes), as this intergrin is selectively induced during angiogenesis. We quantified αvβ3-integrin in vivo, using a RGD-peptide which selectively binds to αvβ3-integrin. Mice were sensitized and challenged with TNCB to induce and elicit CHSR. Twelfe hours later we injected animals either [^{18}F]Galacto-RGD or [^{125}I]Gluco-RGD peptide and scanned mice in vivo with the small animal positron emission tomograph MADPET or determined uptake by autoradiography. In vivo MADPET images showed intense RGD peptide uptake in C-CHSR but not in A-CHSR. This was specific as unlabeld RGD peptide completely blocked the uptake during C-CHSR. Uptake ratio between treated and untreated ear was 2.9 for C-CHSR. In sharp contrast to C-CHSR, [^{18}F]Galacto-RGD binding did not increase in A-CHSR. The uptake of labelled RGD peptide increased linearly with the episodes of inflammation. Immunohistochemistry confirmed that β3 expression increased exclusively in C-CHSR. TNF$^{-/-}$ and IL-4$^{-/-}$ mice had normal αvβ3-integrin expression and developed unaltered angiogenesis in C-CHSR. This suggests that angiogenesis during C-CHSR is independent of these two cytokines. Thus, angiogenesis and αvβ3-integrin plays are critically involved in C-CHSR but not in A-CHSR. Our data represent a new model for quantifying angiogenesis in vivo and thus may help to bring to elucidate the mechanisms underlying T cell mediated inflammation and tissue destruction.

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Up-regulated expression of CXCR3 but not CLA in skin-infiltrating malignant T cells regulates epidermotrophism in CD8-positive Sezary syndrome

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We have previously shown that a T-cell line (CD8SzCL) was established from blisters arising on skin of a patient with CD8$^+$ Sezary syndrome. The malignant cells were CD3^{low}CD8$^+$CD45RA$^+$CD56$^+$ and expressed mRNA for IL-10 and IFN-γ. Biopsies showed marked epidermotropism of the tumor cells. In this study, we further elucidated the mechanisms of migration of the tumor cells. The malignant cells in the PB were CXCR3$^-$, while the cells in the blister expressed it. CXCR3 on PB tumor cells was changed to positive when they were cultured in the medium. CXCR3 expression was reversibly downregulated by the addition of patient's serum to the culture. The tumor cells from both blisters and PB constantly expressed CLA and its intensity was unchanged. In addition, the cells freshly isolated from blisters expressed a high level of granzyme B, while the expression level of this molecule was lower in the PB tumor cells. We next performed chemotaxis assay using a Transwell plate. CD8SzCL (CLA$^+$CXCR3$^+$) showed increases in movement in response to IP-10, but not to CCL27. When cultured patient's PBMC, including CLA$^+$CXCR3$^+$ tumor cells, were placed in the top chamber, the percentage of tumor cells was significantly increased after passage of the membranes, demonstrating selective attraction of the tumor cells by IP-10. Such selectivity was not seen when fresh PBMC were applied. Neither fresh nor cultured PBMC migrated with CCL27 added to the lower unit. These findings suggest that CXCR3, but not CLA, plays a major role for tumor cell migration into the skin. Although the tumor cells are capable of expressing CXCR3 and cytotoxic molecules, they are strongly downmodulated in the PB. Once the cells infiltrate into the skin, they bear CXCR3 and migrate toward epidermis by virtue of keratinocyte-derived IP-10. Furthermore, IFN-γ produced by the tumor cells may stimulate the production of IP-10, which might exaggerate epidermotropism.

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Activated neutrophils exert anti-tumor activity against human melanoma cells: reactive oxygen species-induced mechanisms and their modulation by GM-CSF

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Rapid and marked infiltration of polymorphonuclear cells (PMN) into tumors genetically modified to produce cytokines (e.g. GM-CSF) has been observed in several preclinical cancer vaccine models. A similar phenomenon has also been described in melanoma patients after direct intralesional injection of a GM-CSF/vaccinia construct or vaccination with GM-CSF-transduced tumor cells. To date, it is still unclear whether PMN contribute to melanoma rejection by virtue of direct tumoricidal activity. Therefore we established a co-culture model of human melanoma cells with N-formyl-methionyl-leucyl-phenylalanine (FMLP)-activated PMN. Whereas FMLP-activated PMN had no direct effects on the viability of melanoma cells (as determined by MTT-assay), GM-CSF priming of FMLP-activated PMN induced significant dose- and time-dependent reduction of melanoma cell viability with induction of apoptosis. PMN can exert their tumoricidal activity by generation of reactive oxygen species (ROS). We therefore analyzed the release of superoxide anion (SOA) and found significantly enhanced release of SOA after GM-CSF priming of FMLP-activated PMN. Generation of SOA is known to represent the first step in a cascade of potential cytotoxic intermediate-products including hydroxyl radicals. Release of SOA is the first step in a cascade leading to the generation of potential cytotoxic reactive oxygen intermediates including hydroxyl radicals. Direct and indirect inhibition of hydroxyl radical generation with combinations of superoxide anion dismutase and catalase, the hydroxyl radical quencher dimethylsulfoxamide or the nitric oxide inhibitor NG-monomethyl-L-arginine resulted in nearly complete protection of melanoma cells against the tumoricidal effects of cytokine-primed PMN. These results support the assumption that cytokine-primed PMN can exert direct anti-tumor activity against melanoma cells via release of hydroxyl radicals.

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L-selectin and CCR7 expressing T cells represent the minority of skin T cells in normal and diseased skin

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Molecules reported to have key role in T cell homing to the skin include CLA and CCR4. We have previously shown increased expression of the skin homing molecules CLA and CCR4 in the peripheral blood of cutaneous T cell lymphoma (CTCL) patients compared to healthy subjects and showed increased expression of these molecules in both normal and CTCL skin. While CLA and CCR4 play important role in T cell homing to the skin, L-selectin and CCR7 have been shown to participate in T cell entry to the lymph nodes. Although there is data in the literature suggesting that these L-selectin+CCR7+ central memory T cells do not enter tissues, our results indicate that a large percentage of peripheral blood T cells expressing CLA and CCR4 also express L-selectin and CCR7.These T cells have features of both central and effector memory T cells. In this study we analyzed the expresion pattern of the lymph node homing molecules in the blood and skin of CTCL patients and healthy controls. Our results demonstrate that while the majority of T cells in the circulation of both patients and controls express L-selectin and CCR7, skin T cells (normal and diseased)express low levels of these molecules. This is in contrast to the expression pattern of CLA and CCR4, which are expresed on a small subset of blood T cells and the majority of skin lymphocytes. Our data indicate that the very high ratio of lymph node/skin homing molecules found in the peripheral blood (normal and CTCL)shows an inverse pattern in the skin.Interestingly, particularly low levels of L-selectin and CCR7 co-expressing T cells were detected in CTCL skin lesions even when most malignant T cells expressed these molecules in the blood. These data suggest that from the pool of circulating T cells, the majority of which express lymph node homing molecules, the L-selectin/CCR7 negative, CLA/CCR4 positive T cells are recruited to the skin. Alternatively, it is also possible that L-selectin and CCR7 are downregulated in the skin microenvironment.

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NKT cell subsets in the peripheral circulation of patients with psoriasis

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Despite evidence of the presence of NK, NKT and T cells in psoriatic plaques, the specific lymphocyte subset(s) responsible, their regulation and mechanistic role remain unresolved. Following the recent identification of two stably expressed cell surface markers, IL-18R and ST2L, which are selectively expressed on the surface of Type 1 and Type 2 NKT subsets respectively, we were interested to determine how these subsets were distributed in patients with inflammatory skin disease. The ratio of Type 1 and Type 2 lymphocytes in PBMC of patients with mild to severe psoriasis (n=20) or atopic eczema (n=14) were examined by three colour flow cytometry for cell surface expression of ST2L or IL-18R together with CD3 or invariant TCRa chain Va24JaQ and CD4, CD8, CD56 or CD161. There was no significant difference in the proportion of CD4+ or CD8+ T cell subsets in patients with psoriasis compared to those with eczema or to normal controls (n=10). In contrast, NKT cells expressing CD161 and TCR Va24 were significantly increased in patients with psoriasis compared to healthy controls and to those with atopic dermatitis. Importantly, we found a predominance of STL2+ NKT2 cells over IL-18R+ NKT1 cells in PBMC of patients with mild to moderate psoriasis ($N = 12$, $p = 0.005$) but not in normal individuals or patients with atopic dermatitis. In contrast, there was a significantly higher proportion of Type 1 to Type 2 NKT cells in people with severe psoriasis ($N = 8$). These observations suggest that NKT cell subset imbalance may play a role in the pathogenesis of psoriasis. Interestingly, NKT2 cells typically co-express IL-4 and IFNγ, two cytokines previously identified in T-cells derived from patients with psoriasis. Moreover, they can bind to the non-classical MHC-like CD1d antigen that is known to be expressed by keratinocytes in lesional plaque psoriasis. The ability to distinguish functional NKT-cell subsets will be invaluable in further defining the role of these cells in the pathogenesis of psoriasis.

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***Propionibacterium acnes* induces antimicrobial pathways through toll-like receptor 2 in human neutrophils**

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One of the factors that contribute to the pathogenesis of acne is Propionibacterium acnes. Although the entire mechanism by which the immune system defends against this pathogen is not understood, it is believed that innate immune system plays a critical role. Neutrophils, an integral part of innate immunity, control infection through a number of different antimicrobial mechanisms including the release of cytokines, reactive oxygens, and antimicrobial peptides such as Human Neutrophil peptide (HNP). In acne lesions there are abundant neutrophils infiltrating around the pilosebaceous follicles at the site of the disease suggesting that neutrophils play a role in host defense. Recent studies have demonstrated that granulocytes express Toll-like receptors (TLRs) and mediate responsiveness to a variety of molecular structures from microbial pathogens. Therefore, the present study was devised to elucidate the mechanism by which P. acnes induces antimicrobial activity in neutrophils. Using confocal microscopy, we found that neutrophils are abundant in acne lesions and that they expressed TLR2. Using human peripheral blood, we isolated neutrophils and demonstrated that they expressed TLR2. We found that these neutrophils could be activated by P. acnes to induce the release of reactive oxygens and HNP. Interestingly, the release of reactive oxygens could be blocked by using anti-TLR2 antibody. Our data suggest that TLR2 is the direct mediator of signaling by P. acnes in neutrophils and may initiate antimicrobial response of neutrophils against P. acnes. As such, TLR2 agonists may provide a novel target for treatment of this common skin disease.

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Development of a unique CD4+ T cell phenotype via α5 integrin modulation of CD3/CD28 signaling

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We previously showed that activation of CD4+ T cells by tolerogenic macrophages infiltrating UV-exposed human skin (UV-Mph) differs from activation by Langerhans cells (LC). This includes deficient expression of IL-2 receptor alpha chain (CD25) and induction of Th2 cytokines by the UV-Mph. Relative to LC, UV-Mph while deficient in their expression of B7-1 and 2, nonetheless stimulate T-cell proliferation. Blocking T-cell α5 integrin (A5) inhibits UV-Mph, but not LC mediated T-cell growth. We asked whether A5 signals can compensate for deficient CD28 signaling and provide synergistic costimulation to activate T-cells, such as is induced by tolerogenic UV-Mph. We now characterize this unique CD4+ T-cell by in vitro activation. To model activation by either LC (anti-CD3/high anti-CD28) or UV-Mph (anti-CD3/ anti-A5 with low anti-CD28) Ag presentation, purified CD4+ T-cells were added to antibody coated plates. With equal T-cell proliferation, CD69 was equivalent while CD25 expression was reduced 30% in the UV-Mph model. Th1/Th2 cytokine array of Il-2, Il-4, Il-6, Il-10, IFNγ and TNFα analysis showed that the LC model produces a robust cytokine production that favors a Th1 type profile, while cytokine production in the UV-Mph model was reduced 30 to 50%. Further, in the UV-Mph model IL-10:IFNγ was twice the LC model, indicative of Th2 skewing. Subsequently, we examined other markers of T-cell differentiation and found that the UV-Mph model exhibited twice the ccr4 (associated with both Th2 and regulatory cells) and half the CD40L (associated with Th1 cells) surface expression. These results demonstrate that activation of T-cells through A5 can result in a distinct phenotype that is relevant to the induction of tolerance by the UV-Mph; reduced cytokine production with a skewing towards a Th2 cytokine profile, and expression of surface markers characteristic of non-Th1 type cells capable of down regulating cell mediated immunity.

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Human keratinocytes and airway epithelial cells express a number of CC and CXC chemokine receptors

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Chemokine receptors are recognized for their role in leukocyte recruitment to tissues under both homeostatic and inflammatory settings. More recently, they have been discovered on non-hematopoietic cells. Their function on these cells extends beyond chemotaxis and cell activation to include proliferation, adhesion molecule expression, organogenesis, and viral entry. Human keratinocytes have been shown to express CCR1, CCR3, CXCR1, CXCR2, and CXCR4 at the protein level. Our purpose was to study the constitutive expression of chemokine receptors on human epithelial cells. The surface expression of CCR1-CCR10 and CXCR1-CXCR5 was characterized on the epidermoid carcinoma A431 cell line, primary human neonatal foreskin keratinocytes (PHFK), and the human bronchial epithelial cell line (BEAS-2B) by flow cytometry. There was statistically significant constitutive chemokine receptor expression on A431 cells (n=3) for CCR9>CXCR4 CCR3>CCR5>CXCR5>CCR1 and PHFK(n=3) for CCR3 CXCR4>CCR9>CCR5>CXCR5>CCR6. In contrast, BEAS-2B (n=7-12; CCR5>CXCR4>CCR9>CCR3) expressed fewer chemokine receptors. The CCR3 expression on human keratinocytes (PHFK; net MFI=35.2±12.5) in contrast to airway epithelial cells (BEAS-2B; net MFI=4.0±0.6), was even greater than that observed on purified human eosinophils (net MFI=24.9±4.0). Eotaxin (100ng/ml x18hr) stimulation of primary bronchial epithelial cells (n=3) enhanced the surface expression of CCR3 suggesting that there may be an autocrine feedback loop. In conclusion, we have identified numerous chemokine receptors on epithelial cells. The expression is more remarkable on cutaneous epithelial cells with CCR3, CCR9, and CXCR4 as the most abundant receptors. Since epithelial cells have been identified as potent sources of the ligands for these receptors it is logical to speculate that this may be an important autocrine regulatory loop. Current studies are focusing on what regulates the expression of these chemokine receptors and on functional studies aimed at identifying the role they play in human disease.

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Identification and characterization of CD43 as a novel ligand for E-selectin on skin-homing T cells

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Memory T cell recruitment to skin is a critical feature of the immune response to pathogen invasion and to immune surveillance of uninflamed skin. Trafficking of T cells to skin has been linked with expression of the cutaneous lymphocyte-associated antigen (CLA), a carbohydrate epitope recognized by the unique monoclonal antibody, HECA-452. We have shown previously that P-Selectin Glycoprotein Ligand-1 (PSGL-1) can be differentially decorated with the CLA epitope to serve as both an E-selectin and a P-selectin ligand on T cells. Using real-time, direct observation of adhesive interactions between selectin-bearing cells in shear flow and Western blots of CLA+ human T cells lysate, we have identified T cell E-selectin ligands with apparent molecular weights of 240 kD, 140 kD, 125 kD, 110 kD and 100 kD. The 240 kD and 140 kD bands correspond to the previously described dimer and monomer forms of PSGL-1, respectively, and support binding via both E-selectin and P-selectin. The 125 kD and 110 kD bands represent two isoforms of the sialomucin CD43 (leukosialin). The biochemical features of CD43 from CLA+ T cells and its properties as a E-selectin specific ligand were confirmed by immunopurification of CD43 and antigen capture flow analysis. The 125 kD CD43 component bears core-2 modified O-linked sialoglycans decorated with the CLA epitope and functions as a ligand for E-selectin, but not for P-selectin. The 110 kD CD43 component, in contrast, is not modified by core-2 glycans, does not bear CLA epitope and does not have selectin ligand function. The identification of CD43 as a T cell E-selectin ligand distinct from PSGL-1 suggests that the regulation of T cell homing to skin is more complex that previously appreciated. Although CD43 shares structural homology with PSGL-1, its expression and post-translational modification pathways are distinct and suggest a unique or synergistic role for CD43 in the trafficking of T cells to skin.

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Heme oxygenase-1 expression in the skin: low expression pattern in malignant skin tumors but high in benign tumors and psoriasis

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Heme oxygenase-1 (HO-1) is a heat-shock protein that is induced by stressful stimuli such as arsenite, hydrogen peroxide, nitric oxide heavy metals organic chemicals and UV radiation. HO-1 over expression in renal cell carcinoma and oral SCC has been reported. But noting is reported about the expression pattern of HO-1 in skin malignant and benign tumors. The expression pattern of HO-1 in above diseases was studied by immunohistochemistry. Thirty three cases of squamous cell carcinoma (SCC), 25 cases of basal cell carcinoma (BCC), 26 cases of actinic keratosis (AK), 32 cases of Bowen's disease (BD), 33 cases of seborrheic keratosis (SK) and 26 cases of psoriasis, were evaluated. Three (9.09%) of the 33 SCC cases, 7 (28.00%) of the 25 BCC cases, 24 (92.31 %) of the 26 AK cases, 21 (65.60%) of the 32 cases of BD, 19 (57.58%) of the 33 SK cases and 26 (100.00%) of the 26 psoriasis cases showed positive results for HO-1. Twenty (60.60%) of the 33 SCC cases, 9 (36.00%) of the 25 BCC cases showed positive-epidermal expression in normal area around the tumor nest and also cells around the tumors. The upregulation of HO-1 expression in pre-malignant, benign and inflammatory skin diseases, suggest an important role for HO activity in skin repair and disease. We hypothesized that the increased HO-1 activity could be a defense mechanism of cells in damaged/inflammed tissues, which might lead to a blockage of the carcinoma/inflammatory process.

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Promastigote and amastigote stage-specific monoclonal antibodies against *Leishmania major*

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The etiologic agents of leishmaniasis include many complex and epidemiological diverse species from the genus Leishmania. Monoclonal antibodies specific to the infective stage promastigotes and amastigotes of Leishmania are needed for accurate, rapid and early clinical diagnosis, and efficacy of drug therapy against Leishmania infection. Female BALB/c, 6-8 weeks old mice were immunized subcutaneously and intramascularly with fusion protein, obtained from 1 x109 promastigotes forms of L. major. The sensitized spleen cells were fused with a mouse myeloma cell line, SP2/0-Ag14, using polyethylene glycol 1500, 3 days after a final booster immunization of sonicated L. major promastigote alone by the intraperitonial route.Hybridomas producing Mab reactive with L. major were screened indirect immunofluorescence staining using L. major promastogotes alone, and differentiated THP-1, J774 cell line infected with L. major. The hybridomas (5x 105) producing Mab (W-2 and W-10) against L. major promastigotes and amastigotes, were injected to SCID mice, intraperitoneal. After 8-14 days mice were sacrificed and peritoneal fluid was collected from peritoneal cavity and processed for purification. Mab efficacy was analyzed by flow cytrometric analysis, western blotting, indirect immunofluorescence staining. On western blotting the Mab (W-10, W-2) showed a positive band at 62 kDa. On indirect immunofluorescence staining the promastigotes showed positivity on parasite surface and cytoplasm. Amastigotes, phagocytosed by macrophages, showed positivity in the cytoplasm. On flow cytometry both antibodies identified intracellular amastigotes and more than 60% cells showed positivity. Monoclonal antibodies are expected to be useful for the diagnosis epidemiology of cutaneous leishmaniasis caused by L. major. Monoclonal antibodies can also be useful to evaluate the efficacy of new drug therapy against L. major.

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Soluble glycoprotein factor/s secreted by *Candida albicans* inhibit human monocytic interleukin 12 production by targeting ERK MAP kinase

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Our previous data have shown that *C.albicans (CA)* inhibition of induce–interleukin 12 (IL–12) production by human monocyte can be mediated by soluble factor/s released from *CA* but not the nonpathogenic *S. cerevisiae*. The data also showed that the soluble factor/s is greater than 30kDa and heat–resistant. To better understand the mechanism underlying this IL–12 inhibition, the mitogen–activated protein kinase (MAPK) signaling transduction pathway was studied. We found that *CA* supernatant markedly induced phosphorylation of ERK1/2 MAPK ($P<0.005$, n=5), but not p38 and SAPK, at 1 min (very early time point). Since interferon–γ (IFNγ, 1ng/ml) plus lipopolysaccharide (LPS, 100ng/ml) were used to stimulate IL—12 production by monocytes, we also observed and found, however, that IFN γ+LPS did not significantly affect supernatant–induced ERK MAPK activity. Specific inhibitor for ERK MAPK, PD98059 (20μM), significantly blocked *CA* supernatant–induced ERK MAPK expression($P<0.05$). Interestingly, Our data also showed that induction of ERK MAPK is inreversely correlated with IL–12 production by monocytes, and that the inhibition of ERK MAPK led to restoration of the IL–12 levels ($P<0.01$). These data indicated that *CA* secretes factors inhibiting human monocytic IL–12 production in which MAP Kinases signaling pathway is critically involved. Additionally, we further characterized *CA* supernatants by treatment of the supernatants with proteinase K (20μg/ml) or alkaline hydrolysis (0.2N NaOH), both led to abrogation of the inhibitory activity relative to their controls: inactivated proteinase K or exposure to blank alkine dialysate, suggesting that the inhibitor/s in *CA* supernatant is proteinacious and glycoprotein in nature. Further fractation and characterization of *CA* supernatants is needed to elucidate the biochemical characteristics of the inhibitor/s.

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Recombinant expression and functional analysis of the protein encoded by Mce1A region of *Mycobacterium leprae*

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Mycobacterium cell entry protein (MceP) is a protein associated with epithelial cell entry, and is only expressed among members of the M. tuberculosis (MTB) complex. On the other hand, it is known that mce1A region of M. leprae has a high degree of homology with MceP encoding sequence. In our new study, the recombinant (r-) Mce1A proteins (45kDa, 37kDa, 27kDa and 19kDa), which were deleted at N-terminus and/or C-terminus amino acid residues of full length 47kDa Mce1A, were expressed and purified using r-E. coli systems and these characteristics were examined. Attachment and entry into epithelial cells was investigated using 45kDa r-Mce1A-coated FITC labeled polystyrene microsphere beads associating with HeLa cells. The attachment and entry of both 45kDa r-Mce1A and MTB r-MceP into HeLa cells were identical as well. In addition, 45kDa r-Mce1A reacted with anti-mouse MTB r-MceP polyclonal antibody. These results suggest that 45kDa r-Mce1A protein has an activity of entry into epithelial cells as well as MTB r-MceP and has an antigenic similarity with MTB MceP. These suggest that the mce1A cording protein is an important virulence factor associated with epithelial cell entry. Since percutaneous thick contact or airborne infection are important infective pathways for Hansen disease, we continue examining the role of Mce1A in the entry of M. leprae into skin and nasal mucosa.

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Epifluorescent microscopic visualization of an *in vitro* biofilm formed by a *Pseudomonas aeruginosa* wound isolate and of an *in vivo* polymicrobial biofilm obtained from an infected wound

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Our lab has demonstrated that wound pathogenic bacteria establish biofilms in vitro, and in acute and chronic infected wounds. To broaden our current understanding of biofilm morphology in wounds, we used epifluorescent microscopy to visualize wound pathogenic bacterial biofilms. In-vitro wound pathogenic Pseudomonas aeruginosa biofilms, and polymicrobial biofilms obtained from chronic infected wounds in humans were examined. Pseudomonas aeruginosa was grown in Tryptic Soy Broth while shaking at 37 C (48 hrs). This sample was smeared onto a slide, fixed with 2.5% formalin and stained with ethidium bromide (1mg/L; Sigma). It was then washed with distilled water, and stained with calcofluor white (75 mg/L). The bacterial DNA, stained with ethidium bromide, was revealed by red fluorescence. The exopolysaccharide (EPS) appeared as a blue fluorescence. A swab smear was taken from a chronic venous leg ulcer and fixed with 2.5% formalin and stained with only calcofluor white that enabled visualization of the wound biofilm EPS. Images obtained using epifluorescent microscopy demonstrate the complex nature of wound pathogenic bacterial biofilms both in vitro and in vivo. The understanding of the histology of bacterial biofilms in chronic infected skin wounds will provide us the fundamentals to determine its role in wound healing.

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Demonstration of *Staphylococcus aureus* biofilms in acute partial thickness wounds in pigs using electron microscopy

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Biofilms are groups of microorganisms that adhered to surfaces and form a polysaccharide matrix. These microorganisms exchange genetic information, nutrients, virulence and growth factors. We have confirmed the presence of biofilm formation in wounds using a modified Congo red stain. The purpose of this study was to examine the formation of biofilms in acute wounds over time using a scanning electron microscopy. Partial thickness wounds were made on the backs of swine. Wounds were inoculated with Staphylococcus aureus or left un-inoculated to serve as controls. All wounds were covered with a polyurethane film. Biopsies were taken from each wound at 0, 24, 48, or 72 hours. The biopsies were processed for scanning electron microscopic analysis using a modified technique. At time 0, wounds that were not inoculated, had no evidence of biofilm formation. Slight biofilm formation from normal flora was seen with wounds that were not inoculated (24, 48, and 72 hours). Inoculated wounds demonstrated a progressive biofilm formation over time with more prominent structure seen at 48 and 72 hours. The benefit of this model will allow the visualization of S. aureus living in biofilms and could be used to assess the efficacy of therapeutic modalities.

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Efficient anti-MRSA barrier and lytic action of nanocrystalline silver dressings: a preclinical, *in vitro* study

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In the light of an increasing problem of drug resistant, nosocomial infections, we have looked at the antimicrobial property and/or barrier function of Acticoat, a three layers wound dressing consisting of an absorbent inner core sandwiched between outer layers of silver coated polyethylene nets. Moisturized with sterile water, the lower nanocrystalline silver site was put on Columbia agar (Biomerieux, Paris, France) plates saturated either with methicillin-resistant Staphylococcus aureus(MRSA) solutions (s, Mac Farland: 0.5), or colonies (c, density: 10^7). After incubating different plates at 37°C for 1 hour (h), 1 day (d), 2d and 3d, respectively, the numbers of colony forming units (cfu) on the Acticoat upper site (upA), lower site (loA) and the Acticoat -covered area of the plate (pA) were delineated. All test plates were further cultured after removal of Acticoat for 3 d to test potential re-colonization at pA (post-antimicrobiotic effect). At every site, Acticoat killed at least 99.99% (-log6) of bacteria after 1d. MRSA reduction to undetectable levels were found at pA of s-MRSA plates already after 1h. With regard to c-MRSA this cidal effect needed 1d. 2d and 3d cultures of s- as well as c-MRSA plates were also MRSA free. Lack of re-colonization during the post-antimicrobiotic test phase confirmed the complete lytic activity of Acticoat. Additional analysis of Acticoat`s barrier property revealed a log6 reduction of bacteria in upA s-MRSA sites, but no reduction in c-MRSA plates after 1h. In 1d as well as 2d, 3d cultures bacteria decreased under detection levels. Based on the absorbent effect of Acticoat, loA sites always presented with the highest amounts of bacteria (s-MRSA: 1h/no reduction, 1d,2d/-log7; c-MRSA: 1h/no reduction, 1d,2d/-log6) with undetectable levels of MRSA in 3d cultures only. Taken together, we were able to show that nanocrystalline silver dressings have the potency to serve as efficient barrier/antimicrobiotic agent against MRSA in-vitro. Further clinical studies have already been initiated.

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The Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen induces expression of Id-1, a critical cell cycle regulatory protein, in human endothelial cells

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The Kaposi's sarcoma (KS)-associated herpesvirus (KSHV, or human herpesvirus 8, HHV-8) is believed to be the etiologic agent responsible for KS. The pathogenesis of this potentially life-threatening neoplasm is complex and unclear, and it is currently unknown how KSHV causes KS. Id-1 (Inhibitor of DNA Binding-1 or Inhibitor of Differentiation-1) is a naturally occurring dominant negative inhibitor of basic helix-loop-helix transcription factors. Id-1 has been shown to inhibit cellular differentiation, promote cell cycle progression, and has recently been associated with immortalization and tumorigenesis. In this report, we demonstrate that Id-1 is expressed at high levels in KS tumor cells both *in vitro* and *in vivo*, but is expressed at relatively low levels in endothelial cells (ECs), the precursor of the KS tumor cell. KSHV infection of the progenitor cells may be responsible for Id-1 induction as KSHV infection of EC *in vitro* results in a 27-fold increase, on average, in Id-1 protein expression under our experimental conditions. Furthermore, we demonstrate that the KSHV-encoded latency associated nuclear antigen (LANA) appears to be involved. Expression of LANA in ECs results in Id-1 induction similar to that seen in KSHV infected ECs. In contrast, KSHV encoded viral cyclin only modestly induced Id-1 expression (average: 5-fold) and KSHV encoded viral FLIP had no effect on Id-1 expression. These results demonstrate the overexpression of Id-1 in KS tumor cells and indicate the KSHV-LANA protein may be responsible for Id-1 induction in ECs. As Id-1 promotes cell cycle progression and has been associated with tumorigenesis in other cell systems, the induction of this critical protein by LANA may be an important mechanism by which KSHV allows infected ECs to escape normal cell cycle regulation ultimately leading to the development and/or progression of KS.

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Distinct distribution of KSHV subtypes and VEGF-R expression in Kaposi's sarcoma in Central Europe

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Classic Kaposi's sarcoma (KS) is relatively more common in Central Europe but AIDS-associated KS even in untreated cases is less frequent (e.g. ~7% in Hungary) than that in Western Europe.To study the underlying pathomechanism of tumorigenesis, genotyping of KS associated herpesvirus-KSHV viral subtypes and co-expression of KSHV latency associated nuclear antigen-LANA and cytokines involved in KS tumorigenesis such as VEGF-R (vascular endothel growth factor receptor) and bFGF (basic fibriblast growth factor) were compared in classic, AIDS-associated, iatrogenic KS and pseudo-Kaposi samples. Presence and diversity of open reading frame K1 of KSHV was studied by nested PCR amplification and direct DNA sequencing of variable regions of K1. In thin section of tumors KSHV LANA was detected by LN53 mcab, VEGF-R by rhFlt-4 mcab and bFGF by rhFGFb mcab using Vectastain immuneperoxidase staining. Permanent KS cell line (KS-imm) was inoculated sc. into SCID mice an the tumors developed were also analyzed. Genetic analysis defined a narrow distribution of KSHV viral subtypes as: A,A1,A1',A3,A4,A1/C3',A1/C6,C,C2,C3,C3/C3', showing a close phylogenetic relationship between subtypes A and C in this geographical region. The majority of samples were clustering as A1'. Where expressed, KSHV LANA could be detected in spindle cells of tumor recesses with nuclear localization. Highest level of expression was found in AIDS-associated KS saple. Co-expression of VEGF-R and bFGF was observed in all cases independently of the presence of KSHV subtypes demostrating that initiating effect of KSHV on cell proliferation is unlikely. KS tumor samples from SCID mice as well as two iatrogenic KS cases and pseudo-Kaposi samples were KSHV negative by PCR. These observations suggest that possible role of KSHV in the pathogenesis of KS may not be the initiation but the maintenance of cell proliferation.

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The role of human papillomavirus in squamous cell carcinomas

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Although the role of human papillomavirus (HPV) in the pathogenesis of cervical squamous cell carcinomas (SCCs) is firmly established, the evidence supporting a similar role in cutaneous SCCs remains speculative. HPV DNA, especially from epidermodysplasia verruciformis (EV) subtypes, has been detected in cutaneous SCCs arising in immunosuppressed individuals, suggesting a pathogenic role for these HPV subtypes; however, results concerning HPV prevalence in lesions from immunocompetent individuals have been mixed. These discrepancies may be due, in part, to differences in the specificities and sensitivities of techniques used to detect HPV in the skin. We used a degenerate nested PCR technique with the capacity to detect a broad spectrum of cutaneous, mucosal, and EV HPV types to detect HPV DNA in cutaneous SCCs (lesional) and in adjacent, clinically uninvolved (perilesional) skin. We found, among 61 immunocompetent subjects with histologically confirmed cutaneous SCCs, that 38 (62%) had HPV DNA isolated from lesional tissue and 28 (46%) had HPV detected in perilesional tissue. The HPV subtypes present were predominantly EV-associated. These results will be compared to those from skin biopsies taken from sun-exposed areas from age and sex-matched immunocompetent control subjects without a history of skin cancer (results of these analyses are pending). The difference in HPV prevalence between lesional, perilesional and control tissue will help clarify the role of HPV in cutaneous carcinogenesis, specifically in relation to the potential for involvement of other co-factors.

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Quantification of Biofilm formation by *Pseudomonas aeruginosa* chronic wound isolates *in vitro*

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Pseudomonas aeruginosa infections are a major threat to patients with chronic wounds and burns. Some strains grow as planktonic cells while others attach to surfaces and form biofilms, a combination of microorganisms and extrapolysaccharides (EPS) matrices. Once biofilms are established, phagocytosis and diffusion of antibiotics are impaired thus facilitating colonization and persistent infections. The purpose of this study was to assess EPS formation of wild type Pseudomonas aeruginosa isolates from chronic leg ulcers. Biofilm forming ATCC isolates were used as controls. Bacterial suspensions in Tryptic soy broth were grown in polystyrene wells and incubated. At 48 hours, the bacteria suspensions were removed and discarded. Wells were gently washed with PBS, air dried and heat fixed. Wells were stained with Huckers crystal violet solution to visualize the EPS. Excess stain was rinsed off with PBS and Tween 80 was added to wells. The stained EPS was removed by sonication, and their optical density quantitated. All strains developed EPS of varying intensities indicating different amounts of biofilm formation. This study demonstrated that chronic wound Pseudomonas aeruginosa isolates vary in their abilities to produce EPS which may in turn affect biofilm formation and therapeutic outcomes in persistent infections.

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Histological evidence of nuclear factor kappa B p65 activation in skin infectious diseases

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The NF-kappa B family consists of a group of inducible transcription factors which regulate immune and inflammatory responses. A number of signal transduction cascades can activate the NF-kappa B pathway to result in the translocation of the NF-kappa B proteins from the cytoplasm to the nucleus where they activate the transcription of specific cellular genes. It would be interesting to know the extent to which NF-kappa B actually is present in the tissues of skin infectious diseases in vivo. Skin biopsy samples from patients with various skin infectious diseases were obtained. We investigated the localization of the NF-kappa B (active p65 subunit) immunohistochemically by light microscopy and examined using a confocal microscope to confirm nuclear staining. The p65 subunit of NF-kappa B was found in the cytoplasm of some inflammatory cells and it was also localized in the nucleus of a small amount of inflammatory cells. Nuclear staining was observed under a confocal microscope. In our study, the nuclear localization of NF-kappa B, which is equivalent to NF-kappa B activation, was clearly seen in skin infectious diseases. The demonstration of the activation of NF-kappa B may be helpful in defining the identity and role of gene products that contribute to pathogenesis of skin infectious diseases.

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Analysis of antibiotic susceptibility patterns from two large cohorts of pediatric impetigo patients: Puerto Rico versus South Africa

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Impetigo, a common skin infection in children, is caused primarily by Staphylococcus aureus and Group A beta-hemolytic streptococci. Isolates from 450 patients in Puerto Rico and 593 patients in South Africa were identified and then tested for sensitivity and resistance to antibiotics. Data was examined for percent resistance to antibiotics for staphylococcus isolated alone and in co-infection with streptococcus. S .aureus isolated alone was most common in Puerto Rico, 52.44%, while co-infection of staphylococcus and streptococcus was most common in South Africa, 55%. In both countries there was an increase in resistance to a number of antibiotics when staphylococcus was found in co-infection with streptococcus. Approximately 95% of the cultures in South Africa were resistant to penicillin versus 50% in Puerto Rico. In Puerto Rico there were higher rates of resistance to erythromycin as compared to South Africa. Oxacillin resistance was also higher in Puerto Rico compared to South Africa. Different antibiotics were tested in the two regions therefore direct comparison was not possible. More information on antibiotic prescribing practices and use in both places is needed to assess the significance of these patterns of resistance and the possible impact on the treatment of impetigo.

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Vaccination with TAT-LACK fusion protein protects against murine leishmaniasis

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Cutaneous leishmaniasis is a major health problem with significant morbidity. An efficacious vaccine does not exist. Protective immunity depends on IFNγ^+, antigen-specific Th1- and/or CD8$^+$ cells. *In vitro* treatment of dendritic cells (DC) with antigens containing the protein transduction domain of HIV-TAT leads to enhanced MHC-class-I- and II-dependent antigen presentation. We utilized recombinant proteins comprised of TAT PTD and the *Leishmania* antigen LACK as a component of a vaccine. Bone marrow-DC were transduced with TAT-containing or TAT-deficient LACK and injected i.d. on d-7 into *Leishmania*-susceptible BALB/c mice. On d0, infections were initiated with *L. major* using high dose ($2x10^5$) as well as low dose ($1x10^3$) inocula. In all experiments, administration of TAT-LACK-pulsed DC was more protective than injection of DC loaded with LACK alone. Furthermore, TAT-LACK + DC-mediated protection was comparable to that achieved with soluble *Leishmania*-antigen + CpG ODN treatment. 7 wks after high dose infection, lesions in TAT-LACK + DC-treated mice were 33% smaller than those in PBS-treated mice. In contrast, LACK + DC treated mice showed a 22% increase in granuloma size. In low dose infections, lesion sizes were decreased by 50% in TAT-LACK- and 2% in LACK-treated mice (10 wks). We also directly assessed the immunogenicity of TAT-LACK after direct injection (8 µg protein i.d.). Co-injection of CpG as adjuvant was necessary to obtain protective immunity, and protection was comparable to responses achieved with TAT-LACK transduced DC. LACK alone was ineffective, even in the presence of adjuvant. These results suggest that priming of *L. major*-reactive CD8$^+$ cells utilizing TAT-LACK enhances protection against disease. These proof of principle experiments establish the basis for a novel vaccine against murine leishmaniasis. Future studies will explore the potential of TAT-LACK as a treatment for, as well as a therapeutic vaccine against, the infectious disease.

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***In vitro* antifungal activity of membrane-active peptides against dermatophytes and yeasts using broth microdilution method**

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The recent emergence of fungal infections with resistant strains has stimulated the development of antifungal drugs with different mechanisms. Recently, novel membrane-active peptide (MAP)s have been developed and reported to inhibit the growth of various pathogenic fungi at concentration of 2 to 32µg/ml. We examined the susceptibility of MAPs to dermatophytes and yeasts using a modification of the NCCLS M27-A broth microdilution method, and compared the minimal inhibitory concentration (MIC)s of MAPs with those of terbinafine and fluconazole. The MICs of MAPs for dermatophytes ranged from 1 to 10µg/ml. Terbinafine inhibited the growth of most species at the very low concentration under 0.005µg/ml. The MICs of fluconazole were over 0.5µg/ml for all strains. Obviously MAPs have fungistatic activity, although they could inhibit the fungal growth at the relatively higher concentrations than terbinafine and fluconazole in our experiment. This fact suggests the possibility that MAP may be a candidate for the new antifungal agent in the future.

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Decline of Th2 response in senescence can lead to Th1 response in *L. major* infected BALB/c mice

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Ageing is associated with an altered immune response and increased susceptibility to infections. The senescent immune system shows e.g. dysregulated macrophage functions and deficits of T cells, incl. a decrease in mounting a Th2 response. We wondered if in experimental leishmaniasis this decrease would lead to a less severe infection in aged BALB/c mice, or if changed macrophage functions would lead to deterioration in resistant C57Bl/6 mice. When comparing bone marrow-derived macrophages from BALB/c or C57Bl/6 mice there was no significant difference in NO production and phagocytosis or killing of L.major between 2-month old and 18-month old (=senescent) mice. After infection of C57Bl/6 mice in vivo, aged and young mice showed a resistant course. However, in BALB/c mice ulceration of infected footpads progressed slower in aged mice and, surprisingly, in 6 of 8 aged mice ulcers healed. These mice also showed low or absent dissemination of L.major into visceral organs, and their CD4+ T cells released Th1 instead of Th2 cytokines in response to L.major Ag. When looking at possible reasons for this reversed immune reaction we found that macrophages from aged, but not from young BALB/c mice revealed constitutive release of low amounts of IL12. Also, when ageing mice were kept in conditions free of specific pathogens (SPF) they did reveal decreased parasite load after infection, but no more Th1-determined resistance. We conclude i) that in ageing mice a decreased ability to mount a Th2 response can lead to emergence of a Th1 response in BALB/c mice, while the immune response in C57Bl/6 mice remains unchanged, ii) that constitutive release of IL12 in ageing mice may favour the decline of Th2 response, and iii) that for full reversal into a Th1 response during ageing the immune system may need to be continuously stimulated, e.g. by exposure to microbes.

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Involvement of granulysin-producing T cells in the development of superficial microbial folliculitis

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Folliculitis is a common skin disease with inflammation of the hair follicle. Staphylococcus aureus and S. epidermidis are often cultured from the lesions. In the previous report, we showed that human beta-defensin 2 and human neutrophil peptides were expressed in superficial folliculitis. These observations indicate that these antimicrobial peptides play an important role in innate immunity against bacterial invasion. In addition to neutrophilic infiltration, many CD45RO+ T cells were infiltrated in the lesion of folliculitis. However, the role of lymphocytes in the infectious skin disease is not well investigated. Granulysin, an antimicrobial protein expressed by cytolytic T lymphocytes and NK cells possesses lytic activity against a broad range of microbes including S. aureus. In the present study, we assessed the localization of granulysin in the lesional skin and the cell types producing granulysin. Immunoperoxidase labeling of seven samples of superficial folliculitis was performed using mouse anti-granulysin monoclonal antibody (a kind gift from Dr. RL Modlin, Los Angeles, CA) and anti-human antibodies against CD markers. Double-immunofluorescence was also performed and secitons were examined by confocal laser microscopy. Cells positive for granulysin were abundant in pustules and perilesional dermis of all tested samples. We identified CD3+, CD4+ and CD8+ T cells in the lesions. CD20+ and CD56+ cells were not observed. Confocal laser microscopic examination indicated that the granulysin-producing cells were of CD3+, CD4+ T cells but not CD8+ T cells. Our observations indicate that granulysin produced by T cells may play a pivotal role in host defense against microbial invasion in the lesions of superficial folliculitis.

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Detection of human papillomavirus in callus of diabetic foot ulcers by polymerase chain reaction method

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Callus is an important risk factor in the development of diabetic neuropathic foot ulcers. The proposed mechanism for callus formation involves the pressure and stress that occur in the diabetic foot as a result of neuropathy. Moreover, once formed, the callus contributes to skin breakdown and ulceration by increasing the pressure at the site. However, the observation that callus often forms in seemingly compliant patients and despite appropriate off-loading has led us to evaluate alternative pathophysiologic explanations. Since calluses in the diabetic foot often clinically and histopathologically resemble lesions induced by HPV, we have hypothesized that HPV may play a role in the formation of calluses in diabetic patients. Tissue samples were obtained by shave biopsy of callus from the edge of diabetic foot ulcers. DNA was extracted from 11 tissue samples from 6 patients for PCR amplification, using primers that cover HPV-1, 2, 3, 4, 6, 10, 11, 16, 18, 28, 29, 41, 48, 50, 57, 60, 63, and 65. RESULTS: No HPV DNA was detected in any of the samples, and the validity of these observations was confirmed with positive and negative controls. HPV infection is unlikely to be the cause of callus formation in diabetic foot ulcers. Other etiologies may need to be pursued. No previous publication has addressed this issue using PCR methodology. The key to the validity of this study is the choice of the primers, which have detected HPV in multiple studies, and therefore proven to be sensitive and specific. However, the possibility of an unusual type of HPV dominating in diabetic foot, and/or HPV causing only a small fraction of the diabetic calluses, cannot be ruled out completely.

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The dominant-negative HSV-1 recombinant CJ83193 can serve as a safe and effective vaccine against wild-type HSV-1 infection in mice

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Despite the availability of antiviral therapies, the spread of herpes continues to increase worldwide. The use of HSV replication-defective viruses and neuroattenuated mutants as vaccines has been evaluated. The ability of these vaccines to establish life-long latent infection and the fact that they are replication competent when encounted with the wild-type HSV, raise concerns about their safety. We recently generated an anti-HSV-specific HSV-1 recombinant CJ83193, which can inhibit its own replication, as well as the replication of wild-type HSV-1 and -2 in co-infected cells. In this study we examined whether CJ83193 can serve as a vaccine agains t HSV-1 in mice, as compared to wild-type HSV, strain KOS and ICP27 deletion mutant D27. We demonstrate that CJ83193 cannot initiate acute productive infection in the corneas of infected mice, nor can it reactivate from the trigeminal ganglia of mice latently infected by CJ83193 in a mouse ocular model. Immunization with CJ83193 prevents the development of keratitis and encephalitis induced by corneal challenge with wild-type HSV-1 strain mP and reduces the yields of challenge HSV in the eyes and trigeminal ganglia significantly. Moreover, Mice immunized with CJ83193 are capable of developing strong HSV-1 neutralizing antibodies at levels similar to KOS immunized mice, 4-8 fold higher than titers induced by D27, and induces cell-mediated immune response at levels similar to mice immunized with KOS. Antibody titers and immune response are detectable at high levels up to six month, while response induced by D27 is reduced six month after vaccination. These results shed new light on the development of safe and effective HSV viral vaccines that encode a unique safety mechanism capable of inhibiting its own replication and replication of wild-type virus.

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Cathelicidins are effector molecules in the direct antimicrobial action of keratinocytes

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Cathelicidins (caths) are antimicrobial peptides vital to the innate immune response of skin. Caths are made by keratinocytes during inflammation and are also deposited at sites of injury by neutrophils. This investigation sought to determine how keratinocytes store and process caths, and whether the expression of caths by keratinocytes is necessary for skin antimicrobial function. Cath localization was evaluated using immunocytochemistry and confocal microscopy with polyclonal antibodies against either the prepro- cathelin domain or the antimicrobial C-terminal peptide. Cath function was determined by analysis of microbial killing following coculture of keratinocytes with bacteria. In cultured undifferentiated HaCaT cells and primary keratinocytes, human cath (LL-37) was distributed in a perinuclear pattern and localized in the Golgi apparatus. In whole epidermis, mouse cath (CRAMP) was detected in lamellar granules by immunogold electron microscopy, and by immunoblot in lamellar granules isolated by metrizamide density gradient fractionation. Coculture of keratinocytes with heat-killed, TRITC-labeled E. coli BioParticles, followed by immunostaining and confocal microscopy, showed intracellular colocalization of bacteria with caths. Analysis of survival of Staphylococcus aureus, particularly the cathelicidin-sensitive mprF strain, suggested that keratinocytes from wild-type mice kill S. aureus more efficiently than those isolated from cath knock-out mice. This effect was rescued by adding synthetic CRAMP. Further, inhibition of cath activation by protease inhibitors reduced the ability of keratinocytes to kill S. aureus. Thus, keratinocytes phagocytose bacteria and use caths as a mechanism for direct antimicrobial action. Caths present at basal levels in cultured keratinocytes are capable of killing bacteria, and storage of cath in lamellar bodies may facilitate release in vivo. This process enables proteolytic cleavage of stored cath to active antimicrobial peptide, an essential event in the innate immune defense of skin.

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NF-kB activation by *Candida albicans* in human keratinocytes: possible involvement of toll-like receptor (TLR) 2 but not TLR4

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The epidermis of the skin is largely composed of keratinocytes which does not only play an important structural part in forming a physical barrier to foreign microorganisms but also are important functionally in mediating cutaneous immune reactions. Candida albicans (CA), a most common cause of superficial candidiasis, induces cytokine productions from keratinocytes. No detailed interaction between CA and human keratinocytes has been studied previously, but it may involve activation of NF-kB, a transcriptional activator of multiple host defense genes involved in immune and inflammatory responses. Here, we show that CA activates NF-kB in normal human epidermal keratinocytes (NHEKs). Interleukin-8 production by NHEK stimulated with CA was increased in time-dependent manner. Westernblot analysis revealted that CA induced IkBa phosphorylation and degradation in NHEKs. Dual-luciferase reporter assay revealed that CA activated NF-kB and that the activities were blocked by inhibitors against IkB kinase (IKK) and NF-kB. Furthermore, we show that toll-like receptor (TLR) 2 but not TLR4 may be required for CA induced NF-kB activation. After stimulation with CA, TLR2 mRNA expression was up-regulated. However, we failed to detect TLR4 mRNA expression in NHEK with or without CA stimuli. Also, anti-TLR2 antibody inhibited IL-8 production by NHEKs with CA stimuli. Taken together, our results demonstrate that CA activates NF-kB via TLR2 but not TLR4 in NHEKs.

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Peptiodoglycan up-regulates expression of various components of the microbial recognition receptor complex, TLR2, TLR4, CD14, and CD11b/CD18 in human and mouse keratinocyte cell lines

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Peptidoglycan (PGN) is an essential cell wall component of all types of bacteria and thus is an excellent target for recognition by the innate immune system. PGN induces clinical manifestations of infection by activation of macrophages leading to secretion of cytokines. Toll-like receptor (TLR)2 and CD14 are well known to be primary signal-transducing receptors for PGN in macrophages. However, little is known about the response of keratinocytes to PGN. By using the human macrophage cell line, U937, as a positive control, expression and regulation of microbial receptors and cytokine production following 24 hr stimulation by PGN were investigated in human keratinocyte cell lines, A431, HaCaT, CL-22, and mouse keratinocyte cell lines, Balb/MK, PAM 212. PGN increased cytokine production and induced upregulation of TLR2 and TLR4 in human and mouse keratinocytes. Because TLR4 is well known to be a primary signal-transducing receptor for lipopolysaccharide (LPS), cells were stimulated by PGN in the presence of polymyxin B, a well-known lipid A-neutralizing agent to exclude possible LPS contamination effects. Although Polymyxin B blocked the ability of LPS to stimulate cytokine production, it did not inhibit PGN stimulation in keratinocytes. In addition, antibody blocking of TLR2 decreased PGN-mediated cytokine production in keratinocytes, whereas blocking of TLR4 had no effects on cytokine production. Therefore, these data indicate that PGN stimulation occurs through TLR2, but TLR4 upregulation by PGN might be induced indirectly by TLR2 signaling in keratinocytes. In addition to TLR2 and TLR4, CD14 and CD11b/CD18 were also upregulated by PGN stimulation. Antibody blocking of CD14 and CD11b/CD18 also reduced cytokine production in keratinocytes. Our results suggest that PGN stimulation in keratinocytes is mediated through the activation and cooperation of microbial receptors within this receptor complex.

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Susceptible gene analysis of herpes zoster and postherpetic neuralgia within HLA region

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HLA region is known to have an important role in the immune response against foreign antigen in patients with infectious diseases. In the present study, we have analyzed polymorphisms of HLA genes and 11 sets of microsatellite markers located in the HLA region to investigate precise location of the susceptibility gene for herpes zoster and postherpetic neuralgia (PHN). A total of 162 Japanese patients with herpes zoster (including 102 patients with PHN), 52 patients without herpes zoster and 136 healthy controls were genotyped by the sequence based typing (SBT) method for the class I loci (HLA-A, B, C), and the PCR-restriction fragment length polymorphism (PCR-RFLP) method for the class II loci (HLA-DRB1, DQA1, DQB1). We recognized (1) positive associations of the development of PHN with the HLA class I and II antigens HLA-A*3303,B*4403,DRB1*1302 haplotype, (2) negative associations of the development of PHN with the HLA class I antigen HLA-B*4001 and (3) negative associations of the development of herpes zoster with the HLA class I antigen HLA-B*5101. Interestingly, these associations were recognized also by microsatellite analysis as their markers located around HLA-B were significantly associated in each patients group compared with 248 healthy controls. These findings suggest that the susceptibility gene of PHN and herpes zoster might existed in the HLA class I region rather than class II.

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The role of DC-SIGN in HIV sexual transmission

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Since new HIV infections occur primarily through sexual transmission, small amounts of virus have to cross the mucosal barrier and reach replication-competent sites. Evidence has accumulated that a specific cell subtype, called Langerhans/dendritic (DC) cells, plays a crucial role in the early events of entry into the host (reviewed in Piguet and Blauvelt, JID, 2002). Th receptor DC-SIGN at the surface of DC can capture small amounts of virus in the periphery and transfer it efficiently to T cells, where massive viral replication occurs. Our results demonstrate that DC-SIGN, a receptor that binds HIV with a higher affinity than CD4, is rapidly internalized from the cell surface of DC and targets antigens to the lysosomes, where they are degraded. Furthermore, DC-SIGN internalizes antigens for presentation to T cells. However, HIV is able to escape DC-SIGN-mediated antigen presentation, by dissociating from this receptor early after internalization from the cell surface. In absence of viral replication, this pool of virus is stabilized inside a DC for up to 10 days, whereas free virus in medium looses infectivity in less than three days. We will demonstrate where the virus resides for prolonged periods of time and remains highly infectious with a combination of morphological and virological methods. In order to disturb the early events of HIV infection and the pool of virus hiding in DC, we have developed lentiviral vectors expressing stably small interfering RNA (siRNA) targeting DC-SIGN. Our results demonstrate that siRNA targeting DC-SIGN can suppress up to 97% of the surface expression of DC-SIGN. Using this tool we are analyzing the contribution played by this receptor in the overall pathogenesis of HIV early steps of infection. Furthermore, lentiviral vectors expressing stably siRNA that inhibit DC-SIGN expression could prove to be a novel weapon against HIV infection.

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T cell-mediated induction of Epstein-Barr virus oncogene expression in patients with hypersensitivity to mosquito bites and EBV-infected NK cell lymphocytosis

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NK cell lymphocytosis associated with Epstein-Barr virus (EBV) infection often shows severe hypersensitivity to mosquito bites (HMB) characterized by intense local skin reactions and systemic symptoms such as high fever, lymphadenopathy, and hepatosplenomegaly. We previously demonstrated that CD4+ T cells of the patients proliferated well in response to mosquito salivary gland extracts and played a key role in EBV reactivation from latent state in NK cells. Here we investigated whether CD4+ T cells have any influence on EBV oncogene expression in patients' NK cells and immortalization of these cells. Peripheral blood mononuclear cells were taken from the 5 cases of HMB, and purified CD4+ T and NK cells were cocultured in the presence of mosquito salivary gland extracts (4ug/ml). Before and 12, 24, 72, and 120 hours after starting coculture, viral oncogene expression was assessed by RT-PCR and immunostaining. In result, expression of the oncoprotein LMP1 was remarkably increased at 12-72 hours, while another oncoprotein EBNA2 expression was not detected. In contrast, no or little change of LMP1 expression was observed in coculture without mosquito extracts. Furthermore, to investigate whether humoral factors of the CD4+ T cells can induce LMP1 expression, we transferred the supernatants from the culture of CD4+ T cells stimulated with mosquito extracts into the NK cell culture. We observed an increase of LMP1 mRNA expression in the NK cells and proliferation of these cells. These results suggest that the humoral factors from CD4+ T cells stimulated with mosquito extracts can induce expression of oncoprotein LMP1 in the NK cells and is involved in NK cell lymphocytosis.

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Selective proliferation of Epstein-Barr virus-infected NK cell clones in the cutaneous lesions of hypersensitivity to mosquito bites

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Hypersensitivity to mosquito bites(HMB) is a disease of Epstein-Barr virus (EBV)-associated NK/T lymphoproliferative disorders, characterized by skin ulcers, high-grade fever, liver damage and lymphadnopathy with the subsequent development of lymphoma. In the current study, we investigated cytological and genotypic profiles of the circulating and skin infiltrating EBV+ NK cells in 2 patients with HMB and an established EBV+ NK cell line, using immunohistological and molecular probes. EBV+ NK cells determined by the presence of EBV-encoded small nuclear RNAs (EBER) were increased in number up to 50-55% of the peripheral lymphocytes, and infiltrated in the skin, bone marrow and lymph nodes. The NK cells showed surface phenotypes of CD11b+,16+,56+ in case 1, and CD11b+16-56+ in case 2. Many cytotoxic T-cells (CTLs) expressing TIA-1 and granzyme B were present in the skin lesions. Southern blotting studies using an EBV-terminal repeat probe demonstrated no clonal proliferation of EBV+ cells in the peripheral blood or bone marrow in both cases, but 2 distinct EBV+ NK cell clones were detected in the different cutaneous lesions in case 2. An EBV-infected NK cell clone (NOY-1) established from the peripheral blood was of the same origin as one of the 2 clones detected in the cutaneous lesions. The NOY-1 cells were capable of producing IFN-γ, possessed 7.5 copies of EBV-DNA per cell as determined by quantitative PCR, and expressed only EBNA-1 by usage of Qp promoter without LMP-1 or any other EBNAs, suggesting latency I or II infection pattern. These findings indicate that patients with HMB have oligoclonal EBV+ NK cells in the peripheral blood in which selective clones become dominant in the skin following mosquito bites. Although CTLs are infiltrated in the skin lesions, EBV+ NK cells may minimize the expression of the latency-associated viral antigens which might favor cell survival, evading the host immune surveillance.

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Autoreactive T cells and HSV DNA transport in HAEM pathogenesis

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HSV DNA transport to the skin and restricted TCR repertoire usage are significant factors in HAEM pathogenesis. To determine the HSV-specificity of T cells in HAEM lesions, epidermal T cells were cultured with autologous PBMC (10^6/well) and HSV antigen (400μg/ml). Single cell clones were established by limiting dilution in medium with PHA-P (200ng/ml), human IL-2 (40U/ml), IFN-γ (25ng/ml) and GM-CSF (25ng/ml), autologous PBMC and HSV antigen. T cell lines/clones from HSV patients proliferated in response to HSV antigen. T cell lines from HAEM patients were HSV-specific only at 30 hrs after lesion onset, and only 4/25 (16%) clones recognized HSV antigens. Lines/clones established from HAEM patients at 4-6 days after lesion onset recognized cellular antigens, suggesting that they are autoreactive and could be induced by molecular mimicry or self-antigens released during the earlier virus-specific inflammatory response. To examine HSV DNA transport, PBMC from 8 HAEM and 8 HSV patients were fractionated with the Direct CD34 Progenitor Cell Isolation Kit, infected with HSV and cultured without or with GM-CSF and IL-4 to stimulate $CD34^+$ cell differentiation into immature dendritic cells (DC). They were assayed for HSV DNA (by PCR) on days 1-7 in culture. $CD34^+$ cells were enriched to 82-94% purity and their frequency was similar in HAEM and HSV patients (0.26 ± 0.05% and 0.22 ± 0.1%, respectively). $CD34^-$ populations had 20-40% $CD14^+$ cells. HSV DNA was seen in infected $CD34^-$ cells from both HAEM and HSV patients. HAEM patients differed from their HSV counterparts without HAEM in that their $CD34^+$ cells retained the HSV DNA at 5-7days p.i. The higher stability of HSV DNA was also seen in $CD34^+$ cells from HAEM patients cultured without cytokines or with TNF-α (to generate mature DC). The data suggest that HAEM is a virus-induced disease with an autoreactive component and HSV DNA is transported to the skin by immigrating $CD34^+$ cells.

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Dendritic cells take up *Leishmania* parasites via Fc-receptors: implications for resulting immune responses

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Previously, we demonstrated that *L. major*-infected dendritic cells (DC) - unlike macrophages (MΦ) - release IL-12 and effectively vaccinate against progressive disease. Additionally, we observed that i) skin-DC take up only *L. major* amastigotes, whereas MΦ efficiently phagocytose amastigotes as well as promastigotes, and ii) the kinetics of *L. major* uptake was much slower in DC than in MΦ. These differences could partially be related to differences in the involved receptors. In this study, we initially confirmed that bone marrow-DC (BMDC) - like skin-DC - efficiently take up amastigotes (isolated freshly from infected tissues), but not cultured promastigotes (30±10 vs. 2±1 % infected DC/18 hrs, $p<0.005$). We then analysed the receptors involved in amastigote uptake by BMDC. We have previously shown that uptake of parasites by CD18-(thus CR3-)deficient MΦ is dramatically reduced. In contrast, CR3-deficient BMDC generated from CD18-/- mice showed no alterations in *L. major* phagocytosis. We next studied if opsonization by Ig is involved in *Leishmania* uptake. Amastigotes isolated from B-cell (μMT) or T- and B-cell deficient (SCID) mice devoid of surface Ig were not phagocytosed (3±2 and 4±2 %, $p<0.02$) compared to amastigotes isolated from BALB/c mice. When, however, promastigotes were preincubated with Ig-containing serum or isolated IgG from infected mice, they were efficiently taken up by DC (11±4 %, $p<0.05$). Similar to unopsonized promastigotes, promastigotes opsonized with normal mouse serum were not phagocytosed. Our data suggest, that parasite uptake by DC is dependent on Fc-receptors, whereas MΦ take up *Leishmania* via CR3, and explain why *Leishmania*-infected DC - in contrast to MΦ - release IL-12. Our data may also explain why MΦ phagocytosis of the parasite leads to MHC class II-restricted antigen presentation, and in DC class I and II-dependent presentation of *L. major*-antigens is observed resulting in efficient $CD4^+$ and $CD8^+$ T-cell priming.

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Production of MIP-1α/ CCL3 is mediated via toll-like receptor 3 in human keratinocytes

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MIP-1α is a chemokine that binds to CCR1 and CCR5. MIP-1α induces monocytes and Th1 lymphocytes, and plays an important role in skin immune reactions. It has been reported that TNF-α, IL-1, and IFN-γ cannot induce MIP-1α production by keratinocytes. Interestingly, MIP-1α was detected in vesicular fluids in herpes simplex infection. Recently, we found that Toll-like receptor 3 (TLR3) was strongly expressed in cultured human keratinocytes. Several groups showed that TLR3 is a receptor for virus-associated double-stranded RNA (dsRNA). This led us to hypothesize that keratinocytes synthesize MIP-1α via TLR3 by dsRNA. First, we examined the effect of poly(I;C), a synthetic ligand for TLR3, on normal human keratinocytes. Poly(I;C) induced keratinocytes to express MIP-1α mRNA and produce MIP-1α at 1, 3, and 10 mg/ml in a dose-dependent manner, optimally 4.1 ng/ml at 10 mg/ml. Next, we studied the intracellular signaling pathway of MIP-1α production. We added 3 mg/ml poly(I;C) to keratinocyte culture and examined the effect of inhibitors for the NFκB, JNK, and p38 signaling pathways. Adenovirus vector expressing a mutant IκBα was used to block the NFκB pathway. This mutant IκBα cannot be phosphorylated and prevents translocation of NFκB to the nucleus. The mutant IκBα had no effect on MIP-1α production by keratinocytes. SP600125, an inhibitor of the JNK pathway, also had no effect on MIP-1α production by keratinocytes. However, SB203580 and SB202190, p38 pathway inhibitors, inhibited poly(I;C)-induced MIP-1α production by keratinocytes almost completely. Combined, normal human keratinocytes can produce MIP-1α via TLR3 and the p38 pathway. This is the first report that MIP-1α is produced by keratinocytes.

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Epidermal differentiation regulates the production of innate antimicrobial peptides in the skin

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Previously, we showed that apoptosis signal regulating kinase-1 (ASK1) is an intracellular regulator of keratinocyte differentiation, especially late phase differentiation. We hypothesized that epidermal differentiation regulates innate immunity, since epidermal differentiation is thought to protect the body from the outside environment. In this study, we clarified the role of ASK1 in epidermal innate immunity. Epidermal keratinocytes produce the human antimicrobial peptides beta-defensin-1 (hBD1), hBD2, and hBD3. Direct contact of *S. aureus* with cultured normal human keratinocytes triggered the production of hBD2 and hBD3. Then, we examined the effect of ASK1 on the production of BDs. Transfection of the constitutively active form of ASK1 to normal human keratinocytes using adenovirus vector markedly enhanced hBD1, hBD2, and hBD3 mRNA expression, 2.9-, 22-, and 44-fold compared to controls, respectively, as analyzed by ribonuclease protection assay. This induction was almost completely abolished by the addition of p38 inhibitors: SB203580 and SB202190. Next, we analyzed the role of ASK1 in BD production in mouse keratinocytes obtained from ASK1-/- mice. Direct contact between heat-inactivated *S. aureus* and cultured ASK1+/+ mouse keratinocytes for 18 h enhanced mBD2 and mBD6 mRNA expression, 3.8- and 1.6-fold compared to controls, respectively. In contrast, ASK1-/- mouse keratinocytes did not induce mBD2 or mBD6 mRNA (1.1- and 0.9-fold, respectively). These data demonstrate that activation of ASK1 induces BD production and the absence of ASK1 results in the failure of BD production in response to *S. aureus*. Furthermore, peritoneal injection of bacteria resulted in significantly lower survival rate in ASK1-/- mice than in ASK+/+ mice. In conclusion, this is the first report that the ASK1-p38 MAP kinase pathway plays an essential role in skin innate immunity.

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Lymphatic dysfunction in Kaposi's sarcoma-associated herpesvirus *k-cyclin* transgenic mice

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Vascular endothelial growth factor receptor (VEGFR)-3 is expressed by lymphatic endothelial cells in adults. In addition, KSHV-infected tumor spindle cells within Kaposi's sarcoma (KS) lesions express VEGFR-3, supporting the hypothesis that KS is derived from lymphatic endothelial cells. To further understand KS pathogenesis, we generated transgenic (Tg) mice that express KSHV *k-cyclin* within lymphatic endothelial cells under the control of the murine *VEGFR-3* promoter. *k-cyclin* is a cellular cyclin D2 homolog that is expressed within KS spindle cells. In the Tg line that demonstrated the highest level of *k-cyclin* expression, all heterozygous mice died prematurely within 6 months of age. Both male and female *k-cyclin* Tg mice were noted to be slightly heavier when compared with wild-type littermates (at 16 wks, 26.5±3.2 g vs. 23.7±2.8 g, p=0.076). Rapid and shallow respirations were regularly observed in Tg mice just prior to death. Necropsy of Tg mice revealed 0.25-1.5 ml of chylous fluid within pleural cavities. Histologically, lungs showed diffuse atelectasis and severe congestion. Footpads and ears of Tg mice were edematous and erythematous, and histologically showed dermal edema and extravasated erythrocytes. Other tissues also showed signs of edema. Interestingly, when assessed quantitatively by real-time RT-PCR, *k-cyclin* expression correlated with *VEGFR-3* expression within a variety of tissues, and was highest in lungs. Functionally, defects in lymphatic drainage were observed in *k-cyclin* Tg mice following intradermal injection of either Evans Blue dye or FITC-dextran. In summary, lymphatic dysfunction with premature death secondary to pleural effusion was observed in KSHV *k-cyclin* Tg mice. These mice provide a small animal model for assessing the *in vivo* function of KSHV genes. Thus, they should prove useful in further understanding the pathogenesis of KS and other diseases associated with KSHV infection.

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Role of syndecan and glypican proteoglycans in early events of human papillomavirus (HPV) infection

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Heparan sulfate (HS) proteoglycans (HSPG) are required for attachment of papillomavirus capsids to the cell surface and pseudovirion infection. However, receptor proteins involved in early events of infection have not been identified. To characterize the HS carrying cell surface protein(s) keratinocytes were treated with heparitinase I or PI-specific phospholipase C. Removal of cell surface HS by heparitinase I inhibited HPV16 virus-like particle (VLP) binding in a dose dependent manner, whereas treatment with PI-PLC had no effect suggesting transmembrane syndecans, rather than GPI-anchored glypicans as VLP-binding structure. To directly assess the role of specific HSPG, K562 cells stably transfected with syndecan-1, syndecan-4, glypican-1 or empty vector control cells (G. David) were analyzed for their ability to bind purified HPV16 VLP. Binding was stronger to syndecan-1 K562 cells as compared to the remaining transfectants and was correlated with the total amount of HSPG cell surface expression. In addition, susceptibility for infection was analyzed by in vitro infectivity assays using authentic virions of bovine papillomavirus (BPV) or human papillomavirus (HPV) type 11 isolated from bovine skin and human genital warts. With the appearance of spliced viral mRNA as a readout (RT-PCR) all stable transfectants were permissive for infection with native virions. These results indicate that the amount of HSPG expressed on K562 cell surface determines the capacity for papillomavirus binding and infectivity. Since syndecan-1 is the major HS carrying surface protein of human keratinocytes and specifically induced in basal cells of injured epithelia we propose a model implicating syndecan-1 as the primary cellular receptor in natural papillomavirus infection.

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Virus-like particles of human papillomavirus type 92, a new type isolated from a basal cell carcinoma

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Human papillomavirus (HPV) genomes, in particular DNA sequences related to HPV types found in epidermodysplasia verruciformis (EV), have been demonstrated in malignant and benign skin lesions and in hairs from renal transplant- and immunocompetent patients. An etiologic role of HPV in the development of non-melanoma skin cancer is still unclear. Recently, a novel HPV type, designated candidate HPV92, has been isolated from a basal cell carcinoma. HPV92 showed highest similarity to HPV49 (72%) and phylogenetically was distantly related to other types (e.g. 5, 8) in the B1-group of EV-types. To study functional properties of the HPV92 late region we cloned the L1 major capsid protein ORF into a baculovirus expression vector. Sf9 insect cells were co-transfected with AcMNPV DNA, recombinant baculoviruses were plaque purified and high titer virus stocks generated. By SDS-PAGE and Coomassie staining of whole cell lysates of infected Sf9 cells the L1 protein migrated as a 50-55 kD band similar to the L1 of other HPV types. By western blotting HPV92 L1 was recognized by a polyclonal antiserum raised against HPV5 L1 virus-like particles (VLP) reflecting their deduced protein homology. To provide further evidence for the structural integrity of the L1 protein high-MW complexes from L1 expressing insect cell lysates were purified by density gradient centrifugation and analyzed by transmission electron microscopy. Electron micrographs demonstrated spherical particles indicating that L1 of HPV92 is able to self-assemble into capsomeric VLP. Studies are under way to determine whether immunization with HPV92 VLP can elicit (neutralizing) antibodies to conformational capsid epitopes and whether these cross-react with other cutaneous HPV types. HPV92 VLP might be a useful reagent for serology and prophylactic and therapeutic vaccination strategies aimed against a spectrum of cutaneous HPV types potentially associated with non-melanoma skin cancer.

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Detection of cholera toxin bioweapons via melanophores

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There is a pressing need for methods to detect substances associated with bioterrorism. We demonstrate that melanophores provide a method for the rapid and sensitive detection of cholera toxin from V. cholerae. Melanophores are pigment cells, analogous to human melanocytes, found in lower vertebrates, such as frogs and chameleons. Stimulation of G-protein coupled receptors (GPCRs) on these cells by ligands such as light or hormones, results in alterations in the levels of intracellular second messengers. Changes in the level of these second messengers cause the melanosomes in the melanophores to rapidly aggregate or disperse such that the cells appear to change color between white and black. This color change can be visualized by eye and measured with a plate reader or digital imaging system. The GPCRs can be bypassed by compounds that directly effect G-proteins. One such compound is cholera toxin. This toxin ADP-ribosylates the Gs protein. Ribosylation inhibits the GTP-ase activity of Gs, and stimulates cAMP production. We demonstrate that cholera toxin, in the low nanomolar range, rapidly causes pigment dispersion in Xenopus melanophores. Melanophores can thus be linked to an imaging system to provide a rapid and sensitive indicator for the detection of cholera toxin.

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Single gene encodes dual antimicrobial peptides, multiple functions of the cathelicidin (hCAP18/LL-37)

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As resistance of pathogenic microbes to antibiotics increases, endogenous antibiotic peptides are being considered as a source for new treatments of infectious diseases. Cathelicidins are a class of small cationic peptide antibiotics that are an active component of mammalian innate immunity. Human cathelicidin (hCAP18/LL-37) consists of a conserved prosequence called the cathelin-like domain and a C-terminal peptide named LL-37. To study the role of the conserved prosequence, we produced recombinant cathelin-like domain alone and full-length hCAP18 in E. coli. Since cathelin-like protein is similar to cystatins, a family of cysteine protease inhibitors, we analyzed the effect of the cathelin-like domain on the lysosomal cysteine protease cathepsin L. Cathelin-like protein inhibited cathepsin L proteolytic activity by 46% vs. control. Next, we tested cathelin-like protein for antimicrobial activity using solid phase radial diffusion and liquid phase killing assays. The cathelin-like domain, but not full-length hCAP18, inhibited growth of E. coli and methicillin-resistant Staph. aureus at concentrations of 16-32uM. This activity was distinct from LL-37. To understand how pathogens might overcome this element of innate immunity, we tested the effect of a bacterial proteinase on full-length hCAP18. Group A Streptococcus producing proteinase SpeB (a virulence factor) degraded hCAP18but mutants lacking SpeB did not. Together, these findings suggest that after processing from the full-length cathelicidin precursor, the cathelin-like domain contributes to host defense by inhibition of bacterial growth and limitation of cysteine proteinase-mediated tissue damage. These dual functions of the cathelin-like domain are complementary to the antimicrobial activity of the mature LL-37 peptide. We conclude that human cathelicidin gene encodes at least 2 distinct antimicrobials, and therefore represents an elegant multifunctional effector molecule of skin innate immune defense.

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Exposure to nevirapine in Stevens-Johnson syndrome and toxic epidermal necrolysis

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HIV and AIDS are known risk factors for drug reactions. Nevirapine (NVP), a non nucleoside antiretroviral drug has been already reported as inducing SJS or TEN and we previoulsly raised an alert after having observed 15 cases related to NVP in the first 18 months of a European case control study. We report here the final data of this study after 24 more months of inclusion. Among 441 cases of SJS or TEN enrolled from Apr 1997 to Dec 2001, 36 were known to be infected by HIV-1 (8.1%) They were 12 F and 24 M, aged 21 to 62 years (median 36). 12 SJS, 13 SJS/TEN overlap and 11 TEN. Six of 36 (16.6%) died. 24 of 36 patients (66.7%) had been exposed to nevirapine. All but one received simultaneously other antiretroviral agents but no specific drug combination emerged. Since only 3 of 1505 controls were HIV infected (0.2%) a classical case control analysis was not feasible. We used a case-crossover analysis where each case served as his/her own matched control, by comparing exposure during a case period (one week before onset of the disease) and a control period (3 weeks before). In this analysis, relative risk associated with various antiretroviral drugs was raised significantly for nevirapine (RR=infinite [95% CI: 4.7-infinite]), but not for other non nucleoside reverse transcriptase inhibitors. Intake of NVP was stopped before the probable onset of disease for 4 cases, the day of onset for 7 cases, and after the onset for 13 cases. The later the drug was stopped, the more severe was the disease with 0%, 14% and 46% of patients respectively having detachment of epidermis on more than 30% of the body surface area ($p<0.05$). Considering the high risks of SJS or TEN associated with NVP, the existence of alternative drugs with lower risks, and the incresed severity of skin reactions if NVP is stopped late, we suggest to withdraw nevirapine if any cutaneous eruption occurs in the first month of treatment.

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Interferometric biosensors for diagnostic applications

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Rapid diagnostic aids have considerable potential for improving clinical practice. We have developed a new biosensor that relies on changes in the reflectivity of a silicon substrate, to which a probe molecule is attached. The device is able to specifically detect the presence of bacteria directly from culture, without any intervening lysis or amplification. In the first example, DNAs are used to selectively detect *Pseudomonas aeruginosa*. Two rDNAs specific to *P. aeruginosa* and one rDNA specific to *E. coli* were obtained in 5'-biotinylated form. These were individually spotted on streptavidin-coated silicon wafers. Treatment of these spotted substrates with *P. aeruginosa* in LB for 5 to 45 minutes, followed by thorough rinsing of the chip, led to changes in reflectivity at locations corresponding to the *Pseudomonas*-derived rDNA spots. Treatment with LB media alone, or *E. coli* in LB media, produced little to no change in the reflectivity. In the second example, Tir, an *E. coli* 0157:H7 protein responsible for its pathogenicity, was attached to a silicon surface. When exposed to a solution of purified Intimin, an 0157:H7 protein known to bind Tir, changes in the surface reflectivity consistent with Intimin-Tir binding are observed. Exposure to a culture of *E. coli* overexpressing Intimin produces analogous changes in the surface reflectivity, while exposure to *E. coli* lacking the ability to produce Intimin does not. These results suggest that reflective/interferometric devices using DNA or polypeptide probes may have general utility in the rapid and specific detection of pathogenic organisms.

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In Vivo Evaluation of the Bactericidal Activity of Three Wound Antiseptics on Biofilms of Common Wound Pathogens

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Biofilms, associations of adherent bacteria living in an exopolysaccharide matrix, have been found in wounds. The purpose of this evaluation was to investigate the effect of wound antiseptics on biofilm-living bacteria of common wound pathogens in a pig wound model. Partial thickness wounds were created and inoculated with Pseudomonas aeruginosa and Staphylococcus aureus. Wounds were covered with a film dressing for 24 hours to allow for biofilm formation. The wounds were treated with povidone-iodine, hydrogen peroxide, chlorhexidine or PBS. After 10 or 20 minutes, planktonic or biofilm bacteria were recovered and quantitated following neutralization of antiseptics. P. aeruginosa: Planktonic cells were reduced by 2.1 and 2.9 logs from the control value after 10 and 20 minutes of PI treatment and by 3.1 and 5 logs after chlorhexidine treatment, whereas the cells in biofilms were reduced by less than 1.5 logs after PI and chlorhexidine treatment. Hydrogen peroxide failed to reduce both the planktonic and biofilm bacteria. S. aureus: Planktonic cells were significantly reduced after PI and chlorhexidine treatment at all time periods by 2-3 logs, while biofilm-living bacteria were reduced after 20 minute chlorhexidine treatment by 1.4 logs and 10 or 20 minute PI treatment by 0.9 or 4.6 logs. Hydrogen peroxide did not reduce either planktonic or biofilm bacteria. We concluded that none of the antiseptics we examined were completely effective in-vivo in eliminating planktonic or biofilm-living S. aureus or P. aeruginosa during a short treatment time. Povidone-iodine is the most efficient agent against biofilm-living S. aureus, while biofilm-living P. aeruginosa is much more resistant to the tested antiseptics.

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***Leishmania* infection in murine ears following UVB-induced immunosuppression**

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Leishmania major (*L. major*) commonly involves sun-exposed skin, raising the possibility that the course of infection may be affected by UV immunosuppression. However, UVB irradiation effects have not been addressed in *L. major* models using ear infections to assess outcomes. Therefore, we sought to establish a model using murine ears, which serve as an ideal site for combined infection/irradiation studies. The effect of UVB irradiation was examined in the course of *L. major* infection in the ears of C57BL6/J mice. The ears of mice were irradiated with 120mj/cm2 using Kodacel filtered FS40 sunlamps 48 hours before intradermal injection of $2x10^6$ promastigotes/ear pina. Irradiation was continued every 3 days for 2 weeks (wks) post infection with 30mj/cm2 (53% immunosuppression, as determined by DNFB sensitization and challenge). Ear thickness was measured before infection and weekly thereafter. Infected-only animals developed well defined 2-3mm erythematous nodules on pinas, by 3 wks post-infection. No visible or palpable lesions were noted in either the irradiated-infected or the irradiated-only groups. Ear thickness measurements showed no difference between the irradiated-infected group and infected-only group at 3 or 8 wks post infection ($p>0.1$). However irradiation alone may account for this observation since the irradiated-only group ears were thicker than the infected-only group by 3 wks post-infection ($P=0.002$). By 8 wks post-infection this difference declines ($p>0.1$). During the resolution phase of ear swelling, at 7-8 wks post infection, histopathological exam showed diffuse and deep immune cell infiltration with intra- and extra-cellular parasites in both infected-only and the irradiated-infected groups, but was negligible in the irradiated-only group. Although UVB irradiation does not block immune cell infiltration, it may prevent ear nodule formation. Establishing the combination of *L. major* ear infection and UVB exposure allows for assessment of immunological outcomes.

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***Staphylococcus aureus* infection of primary cultured human keratinocytes: a morphological approach**

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Staphylococcus aureus is the most common cause of skin infection, yet, little is known about the mechanisms that underlie the infectious process. The skin is composed of many cell types of which keratinocytes predominate. In our previous studies using bacterial plate assays, we found that S. aureus adhered to keratinocytes efficiently and rapidly (40% five minutes post infection), invaded the cells poorly (2-4% in 4 hours), and upon invasion replicated intracellularly. To further investigate the interaction of keratinocytes with S. aureus, immnuofluorescence assays were performed on human primary keratinocytes infected with S. aureus RN1, a strain of S. aureus that contains a plasmid that constitutively expresses green fluorescent protein (GFP). Successful penetration of bacteria into keratinocytes was observed by fluorescent microscopy. Inside-outside antibody stains confirmed the presence of intracellular bacteria. GFP-tagged bacteria were seen in the cytoplasm of keratinocytes. Intracellular bacteria were first observed within 30 minutes after infection, and the number of infected cells and the number of intracellular bacteria increased in a time dependent fashion. In general, bacteria entered as singlets or doublets and their number appeared to increase over time, indicative of intracellular replication. To determine whether the intracellular bacteria population increased, infected cells were studied over time by adding lysostaphin, an endopeptidase, to eliminate extracellular bacteria at the end of 4 hours. At 4 hours, infected cells contained few bacteria, mainly singlets and doublets with < 5% containing ten or more bacteria. At 6 hours, most of the infected cells contained clusters of ten or more bacteria. At 24 hours, all of the infected cells were filled with bacteria in the cytoplasm. These results indicated that S. aureus invasion occurs at an early stage of infection followed by replication inside the cells.

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Immediate inflammatory responses to bacterial toxins are reduced, whereas their toxic effects are greatly increased in the absence of mast cells

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Mast cells (MC) are critical effector cells in the induction of protective immune responses to bacteria. Little is known, however, about how MC are activated and how MC provide host protection in the context of bacterial infections. Virulence of bacteria is, at least in part, attributed to bacterial toxins (BT) such as streptolysin O (SLO) from Streptococci or alpha toxin (AT) from Staphylococci. Thus, we asked whether SLO or AT can activate MC and whether MC can downmodulate the toxic effects of these BT. Both SLO and AT dose dependently induced degranulation of murine peritoneal MC as assessed by quantification of serotonin release. In addition, intracutaneous injections of SLO or AT into the ears of C57BL/6 mice resulted in pronounced ear swelling responses within the first hour after injection, suggesting that activated MC had increased extravasation by releasing vasoactive mediators including histamine. Interestingly, genetically MC-deficient Kit^W/Kit^{W-v}-mice injected with BT developed markedly impaired early inflammatory responses as compared to normal Kit+/+ mice. Indeed, SLO only induced ear swelling in Kit^W/Kit^{W-v}-mice that had been reconstituted with MC prior to injection, indicating that MC activation is required for the development of early inflammatory reactions to BT. Most notably, ears of Kit^W/Kit^{W-v}-mice injected with AT exhibited dramatically increased toxic damage, i.e. increased ear thickness on day 10 post injection, as compared to Kit+/+ mice (543±48µm vs. 252±62µm, $p<0.01$). These findings suggest that the early activation of cutaneous MC by BT after infection results in increased extravasation and recruitment of other proinflammtory cells, which helps to protect the host from toxin-induced morbidity. Ongoing investigations will clarify the significance of our findings in skin infections by Streptococci and Staphylococci and may explain how MC limit the pathogenic effects of BT.

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***Staphylococcus aureus* invade and replicate in epidermal and dermal cells**

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Staphylococcus aureus is the most common cause of cutaneous infection. In order for S. aureus to establish skin infection, the bacteria must attach, invade and replicate. However, the skin is composed of several cell types and the relative risk of infection to each cell type is unclear. Therefore, five cell types found within skin (macrophages, Langerhans cells, endothelial cells, fibroblasts and keratinocytes) were grown in culture and tested for their ability to host S. aureus infection. Adhesion and invasion, critical steps for the establishment of an infection, were assayed in all cell types. For adhesion assays, bacteria were co-incubated with mammalian cells for 30 minutes and adherent bacteria were counted. Greater than 50% of input bacteria adhered to all cell types studied. In invasion assays, bacteria and mammalian cells were co-incubated between 30 minutes and 4 hours. Intracellular bacteria were harvested from lysed cells and counted. By one hour, 40-70% of bacteria invaded macrophages and Langerhans cells. In contrast, intracellular bacteria in fibroblasts and keratinocytes were first detected in 4 hours at a level of 2-4% of bacteria. Endothelial cells gave an intermediate phenotype; 20% of bacteria invaded within 4 hours. Interestingly, the proportion of bacteria detected in adhesion and invasion assays was similar regardless of multiplicity of infection. Cytochalasin D, an inhibitor of actin polymerization, effectively blocked invasion in all cell types but had no effect on adhesion rates. Keratinocytes, the predominant cell type found in the epidermis were tested for the ability of bacteria to replicate intracellularly upon invasion. At eight hours post-invasion, bacteria multiplied 50 times in keratinocytes. Similar results were obtained in all cells studied. In summary, we found that all cell types served as efficient hosts and, therefore, should be considered potential targets for S. aureus infection of the skin.

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The Cell Sorted skin equivalent as a model for the study of HPV

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Human papilloma virus (HPV) infections are difficult to model because the viral lifecycle is dependent upon differentiation of the epidermal host cells. The use of raft cultures has been crucial, but limitations in the size and reproducibility of these systems has impaired some studies. Here we show that our novel method of modeling human skin, the Cell Sorted skin equivalent (CeSSE) can be adapted as an effective tool in HPV research. CeSSE relies on the inherent cell adhesive properties of keratinocytes and dermal fibroblasts to sort-out into distinct layers from amixed cell slurry. (J Invest Derm 2000, 114:674). Here using our CeSSE system along with a keratinocyte cell line persistently infected with HPV-18 we demonstrate a model that forms normal human skin that is permissive for viral reproduction. To establish an in vivo HPV activity assay using SCID mice we show that the HUC18 cell line that is persistently infected with HPV18 will generate in vivo CeSSE skin when combined with normal fetal fibroblasts. Positive staining with anti-filaggrin antibodies established these cells as forming morphologically correct skin that sufficiently differentiated to support virus production. We document the presence of the virus in this in vivo CeSSE skin system by immunohistochemical staining for E2, E7, and L1 expression, as well as by electron microscopic detection of virions in the HUC18-CeSSE. Lastly we present initial results demonstrating the use of HPV incorporation into our in vitro self-sorting skin equivalent model, Accuskin. We feel we have successfully developed a working HPV in vivo CeSSE model of virus reproduction that may aid in testing anti-HPV compounds.

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Does survivin play a role in chemoprevention of ultraviolet exposure-mediated cutaneous damages in SKH-1 hairless mouse by resveratrol?

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Ultraviolet (UV) B radiation from the sun elicits many adverse effects on skin and is the major cause of skin cancer; and chemoprevention by naturally occurring agents may be a useful strategy for its management. We earlier showed that resveratrol (*trans*-3, 5, 4'-trihydroxystilbene), a polyphenolic antioxidant present in grapes and red wines, imparts protection from UVB-mediated cutaneous damages in SKH-1 hairless mice. The mechanism of the action of resveratrol is not well-understood. This study was aimed to define the involvement of **Survivin**, a member of the inhibitor of apoptosis protein (IAP) family that is expressed in many cancers, in skin cancer chemopreventive effects of resveratrol. Because multiple UV exposure increases the skin cancer risk in individuals, we evaluated the effect of resveratrol on multiple UVB (180 mJ/cm^2 x 7 in 2 wks) exposures in SKH-1 hairless mice. The animals were subjected to UVB exposures alone or in combination with resveratrol (10μ mole/0.2 ml acetone/mouse), topically applied on mouse-skin prior (30 min) to UVB exposure. The mice were sacrificed at 24 h following the last exposure, and studies were performed. Our data demonstrated that UVB exposure resulted in a significant up-regulation in the mRNA and protein levels of survivin, as assessed by RT-PCR and immunoblot analysis, respectively. Further, resveratrol-treatment was found to inhibit the UVB exposure-mediated increase in bi-fold skin thickness, skin edema and infiltration of leukocytes. Resveratrol treatment was also found to cause a down-modulation in UVB exposure-mediated increases in cyclooxygenase (COX)-2, ornithine decarboxylase (ODC) and proliferating cellular nuclear antigen (PCNA), the known markers of cellular proliferation and tumor promotion. Our data suggested that the chemopreventive effects of resveratrol might be mediated via modulation in the expression of survivin, and the associated events. To our knowledge, this is the first study showing the modulation of survivin as a mechanism of prevention of photo-damage by a chemopreventive agent.

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Oral *Polypodium leucotomos* extract inhibits PUVA-induced phototoxicity of human skin

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PUVA [psoralens and ultraviolet radiation A (UVA, 320-400 nm)] is an effective therapy for over 20 skin disorders, and its clinical use has been limited by problems related to acute phototoxicity and potential long-term carcinogenecity. An extract of the fern plant *Polypodium Leucotomos* (PL) has been shown to exhibit strong anti-oxidant, photoprotective and immunomodulatory properties. We investigated whether oral intake of PL could reduce the acute PUVA-induced phototoxicity on a clinical and histological level. Normal skin of 10 healthy volunteers with skin types II and III was exposed to PUVA with UVA and oral 8-methoxypsoralen (0.6 mg/kg) alone and to PUVA after oral administration of PL (7.5 mg/kg). Erythema and edema of all skin sites was evaluated with a visual scoring system. Biopsies were taken from sites exposed to the 2x minimal phototoxic dose (MPD) without and with PLE after 48 and 72 hours. H&E staining and immunohistochemistry for detection of sunburn cells and CD1a, CD31, Ki 67 and tryptase positive cells was performed. Clinically, PUVA plus PL resulted in a significant reduction of the erythema and edema associated with acute PUVA-induced phototoxicity ($p \leq 0.0004$) and also in a reduction of the development of pigmentation after PUVA when compared to PUVA alone. Histologically, PUVA plus PL showed a significant reduction of sunburn cells ($p=0.05$) and numerical preservation of Langerhans cells ($p=0.007$) and preservation of their morphology, as well as a significant decrease of vasodilation ($p \leq 0.0001$) and dermal mast cell infiltration ($p=0.038$). PL treated skin biopsies also revealed less epidermal phototoxicity. No difference was found in Ki 67 positive cells. In conclusion, oral PL appears to be an effective systemic chemopreventive agent in reducing PUVA-induced phototoxicity. These findings have important implications as they may indicate a beneficiary effect such as reduction of the chance of developing skin cancer in the future.

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Nitric oxide and genetic damage are mediators of ultraviolet radiation-induced immunosuppression in humans

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The aim of this study was to examine the mechanism by which solar-simulated ultraviolet radiation (ssUVR) causes immunosuppression in humans. We studied suppression of recall responses to nickel in allergic human volunteers. NG-methyl-L-arginine (L-NMMA) was used to inhibit nitric oxide (NO) production, 2,2-dipyridyl was used to reduce hydroxyl radical formation, and T4N5 liposomes which contain the DNA repair enzyme T4 endonuclease V encapsulated in microscopic lipid membrane vesicles were used to enhance DNA repair. Each substance was applied to a separate group of 15 volunteers. Skin was irradiated with a 1000W Oriel xenon-arc lamp. Two, six cm squares on the lower back of each volunteer was randomized to receive either the active or base lotion daily for four consecutive days and these squares were subdivided into smaller areas which received different doses of ssUVR. After the final irradiation, nickel patches were applied to each test site for 48 h. Contact hypersensitivity-induced erythema was measured with a reflectance spectrometer 24 h after patch removal. Both antioxidants but not the T4N5 liposomes provided only marginal, significant protection from sunburn. Low, sub-erythemal doses of ssUVR caused local immunosuppression in a dose-dependent manner. L-NMMA and the DNA repair enzymes but not 2,2-dipyridyl protected the immune system from suppression, suggesting that NO and DNA damage, but not iron-catalyzed production of hydroxyl radicals, are involved in UV-induced immunosuppression in humans. Immunostained frozen sections of biopsies from the treated sites suggested that inhibition of UV-induced migration of dendritic cells from the epidermis was responsible for prevention of immunosuppression by both the NO inhibitor and the DNA repair enzymes.

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Attenuation of UVA-induced human skin keratinocytes damage by (-) epigallocatechin-3-gallate (EGCG)

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Cellular effects of UV irradiation are implicated in human skin photoaging. Previous studies indicated that UV irradiation induces generation of reactive oxygen species leading to skin cell damage. EGCG is the major constituent of tea catechin with antioxidant activity. In this study, the protective effects of EGCG on UVA irradiated human keratinocytes (HaCat cells) were investigated with detection of cell proliferation and apoptosis. Expression of matrix metalloproteinases (MMPs) that are responsible for the degradation of collagen knowingly leading to cutaneous aging was also measured. EGCG was applied to the cell culture medium immediately after UVA (1.98 and 3.96 mJ/cm2) and UVB (21, 42 and 126 mJ/cm2) irradiation. The final concentration of EGCG was 0.3, 1.5, and 3 mM. MTT dye assay was used at 24 hours post UV irradiation to determine cell proliferation. Apoptotic and dead cells and bcl-2 protein were detected by FACS analysis. RT-PCR was utilized to measure the expression of MMP1 and MMP3 mRNA. The results showed that UVA and UVB significantly induced apoptosis, inhibited cell proliferation, reduced bcl-2 protein level, and up-regulated mRNA expression of MMP1 and MMP3 in cultured human keratinocytes. In all UV-exposed then EGCG-treated (0.3 mM) groups, cell proliferation, apoptosis rate, and bcl-2 level were restored to the level of non-irradiated groups. The mRNA expression of MMP1 and MMP3 were also comparable to that of unexposed groups. These results suggest that EGCG can be applied to attenuate UV-induced skin cell damage due to its effect on reduction of apoptosis, restoration of cell proliferation and inhibition of MMP expression.

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High DNA repair efficiency in keratinocytes is mediated through strong expression and activation of p53 that induces downstream effectors p48, GADD45, and β-polymerase

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We recently reported that primary human keratinocytes repair DNA photoproducts and oxidative DNA damage 2- to 12-fold more efficiently than primary fibroblasts or melanocytes. This difference was demonstrated in cells from 5 - 17 different donors, including all three cell types from a single donor, and was independent of keratinocyte differentiation. In order to elucidate the mechanism of this difference in DNA repair efficiency, we first determined levels of several DNA repair proteins of the nucleotide- and base-excision repair pathways (NER and BER: XPA, ERCC1, hOGG1, and APE-1) in extracts of primary keratinocytes, fibroblasts, and melanocytes from one donor. No significant differences in expression were found. However, a very strong baseline expression of p53 and activated p53 (serine^{15}-p53) was observed in keratinocytes, while fibroblasts and melanocytes exhibited only weak expression of p53 and activated p53. The high expression of p53 and serine^{15}-p53 was independent of keratinocyte differentiation evident from comparison of keratinocytes cultured in high (1.8 mM), intermediate (0.15 mM), and low (0.05 mM) calcium concentrations. The high expression of p53 and activated p53 provides a very good explanation why both DNA repair pathways, NER for photoproducts and BER for oxidative DNA damage, are more efficient in keratinocytes. In recent years, p53 has been identified to induce both global genome-NER and BER. The effect of p53 on NER is mediated by transcriptionally inducing p48XPE and GADD45, and the effect on BER is mediated by inducing β-polymerase. Consistent with these effects, we found elevated levels of these proteins in keratinocytes, as compared to fibroblasts or melanocytes. The data suggest that keratinocytes have adapted to their increased exposure to environmental mutagens by upregulating excision repair pathways through p53 and its downstream effectors. The data also offer an additional explanation to the central role of p53 mutation in keratinocyte-derived malignancies.

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Aloin treatment attenuates membrane lipid peroxidation and over-expression of cytokines in cultured human dermal fibroblasts exposed to UVA irradiation

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Accumulating evidence indicates that reactive oxygen species generated by ultraviolet irradiation (UV) play important roles in cellular damage leading to apoptosis in human skin. UV irradiation also elevates expression of proinflammatory cytokines such as TNFαand IL-1βwith a result of skin inflammation or sunburn. Aloin has been shown to bear photo protective properties, and yet the molecular mechanisms of its action remain to be elucidated. Using cultured human skin dermal fibroblasts we investigated the protective effects of Aloin on UVA-induced cell damage. FACS analysis with Propidium and Hoechst dye revealed that apoptosis rate increased 38% after UVA irradiation (30 KJ/m2). UVA treatment induced accumulation of Malondialdehyde (MDA) (280 nmol/ml), indicating that UVA-induced damage was associated with lipid peroxidation. Pretreatment with Aloin (10-5M) prior to UVA irradiation reduced apoptosis to 7.5% and the MDA level was reduced to 116 nmol/ml. The results showed that the protective effect of Aloin (10-6 to 10-4M) was dose dependent. RT-PCR results also showed that UVA irradiation caused 2.1 fold increase of TNFαmRNA and 3 fold increase of IL1βmRNA in cultured dermal fibroblasts. Pretreatment of Aloin significantly reduced UAV-induced TNFαand IL1βexpression ($p<0.01$). Taken together, our data indicate that Aloin is an effective inhibitor of membrane peroxidation and can thus be used at appropriate concentration for the prevention of UVA-induced apoptosis, and Aloin may also be applied to prevent UVA-induced skin inflammation.

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***Polypodium leucotomos* extract is an oral photoprotective agent against ultraviolet-induced damage to human skin**

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Exposure of unprotected skin to solar ultraviolet radiation (UVR) induces acute epidermal and dermal damage that can subsequently lead to long-term changes such as photoaging and skin cancer. Currently the most widely accepted method of photoprotection is the usage of topical sunscreens. An oral photoprotective agent acting against photooxidative stress of UVR would have substantial benefits over topical photoprotection in the prevention of solar UVR damage. *Polypodium Leucotomos* (PL) is a natural plant extract endowed with immunomodulating and antioxidative properties, and its oral administration may lead to photoprotection of skin. Ten healthy subjects of skin types II-III were exposed to varying doses of UVR from a solar simulator without and after administration of oral PL (7.5 mg/kg). Clinical evaluation of all skin sites was performed 24 hours (hrs) later. Biopsies were obtained from skin sites exposed to the 2x minimal erythema dose (MED) from PL-treated and non-treated skin after 24 or 72 hrs. Clinically, there was a significant decrease in erythema response in skin exposed within 2 hrs after oral administration of PL ($p\leq0.001$). Histologically, there was a significant decrease in sunburn cells in H&E stained sections ($p=0.03$) and DNA damage analyzed by antibodies against thymine dimers ($p\leq0.0001$) at 24 hrs, and also of proliferation visualized by Ki 67 at 72 hrs ($p\leq0.001$). In total, more than 50% of all biopsies showed Langerhans cells preservation, and this was seen in all biopsies taken at 72 hrs. In conclusion, oral PL appears to be an effective photoprotective agent by decreasing acute UVR induced skin damage such as DNA damage and Langerhans cells depletion. This short-term observation may indicate that oral PL could serve to be effective in the amelioration of short-term UVR damage and this may contribute to prevention of skin cancer or photoaging changes of skin.

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Low dose ultraviolet B irradiation up-regulates thrombomodulin via p38 MAP kinase pathway in human epidermal keratinocytes

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Thrombomodulin (TM), an endothelial cell surface anticoagulant glycoprotein, acts as a cofactor for protein C. TM was initially identified as an endothelial cell surface protein, however it has been also identified in epidermal keratinocytes, especially in Malpighian- layer kerationocyte, and has been recognized as a keratinocyte differentiation marker. It is thought that TM expressed in epidermal kerationcyte may modulate collagen reconstitution of the wound matrix. However, the role of TM expressed in the epidermal keratinocyte are not fully characterized. In the present study, we examined TM expression in response to ultraviolet (UV) B irradiation in immortalized human epidermal keratinocyte cell line, HaCaT. TM expression decreased 2 hours and consequently recovered 16 hours after low dose (100 J/m2) ultraviolet B (UVB) exposure. Message RNA (mRNA) was up-regulated within 1 hour after low dose UVB irradiation. The mRNA of TM decreased with the increasing doses of UVB radiation. A low dose of UVB induced p38 mitogen-activated protein kinase activation rather than c-Jun NH(2)-terminal kinase activation. SB203580, a specific inhibitor of p38 down-regulated TM mRNA level after UVB irradiation and inhibited UVB induced CREB activation. Introducing TM antisense oligonucleotide to HaCaT cells resulted in reducing cell viability after low dose UVB irradiation. Thus, our results altogether suggest that activation of p38-CREB signaling pathway is required for UVB induced TM expression and TM plays important role in early response to UVB irradiation which associated with keratinocyte survival.

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Spectral responses of melanin to UVA irradiation

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Exposure of human skin to UVA can result in pigment darkening. This pigment shows different spectral characteristics compared to normal epidermal melanin or UVB induced pigments. The purpose of this investigation was to study the spectral features of UVA induced pigmant both in an in vitro model system and in vivo. UV-Vis absorbance spectra were measured for dopa-eumelanin and pheomelanin solutions of different concentrations before and after UVA (range from 10-120 J/cm2) exposure. 10 human subjects were irradiated with UVA (40-80J/cm2) on their back and diffuse reflectance spectra were collected for both irradiated sites and normal skin to calculate the spectra of UVA induced pigment. The difference spectra in solution reveals that following UVA exposure the absorbance increases exponentially from 800nm to 450nm in addition to a prominent decrease of absorbance in the UVA range. This change of spectral features depends on both UVA doses and melanin concentrations. The increase of visible absorbance, which may be considered as a marker for new pigment formation, correlates with the decrease of UVA absorbance. Photo-oxidation of pheomelanin solutions presents a distinctively different spectral response as the absorbance in the visible range decreases as well as the absorbance in the UVA range. This change of spectral absorbance is also dose dependent and concentration dependent. In vivo diffuse reflectance spectra show that the UVA induced pigment has a similar spectral characteristic as the in vitro system. For all the volunteers, the increase of absorbance in the visible wavelengths accompanies a fall of absorbance in the UVA range. We conclude that the UVA induced pigmentation produces significant photochemical alterations in the skin witnessed by increased photoprotection in the visible and reduced protection in the UVA. The spectral characteristics are consistent with the in vitro soluble melanin model system, indicating only part of the epidermal melanin may be associated with UVA photodegradation. We also suggest that eumelanin and pheomelanin could be differentiated according to their spectral responses to UVA irradiation.

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UV-induced T suppressor cells inhibit the effector phase of contact hypersensitivity upon local injection

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Epicutaneous application of haptens onto UV-exposed skin induces hapten-specific tolerance. This is mediated via T suppressor cells (Ts), now renamed regulatory T cells, since intravenous (i.v.) injection of T cells from UV-tolerized mice into naive animals renders the recipients unresponsive to the hapten. When T cells from UV-tolerized mice are injected i.v. into mice which were already sensitized, contact hypersensitivity (CHS) is not suppressed, suggesting that UV-induced Ts inhibit the induction but not the elicitation of CHS and are inferior to T effector cells. Since sensitization takes place in the regional lymph nodes, while elicitation in the area of challenge, we postulated that Ts injected i.v. locate to the lymph nodes and not to the periphery and thus only suppress the induction but not the elicitation of CHS. Hence we studied whether Ts inhibit the elicitation of CHS when injected into the area of the challenge. Splenocytes and lymph node cells were obtained from mice which were UV-tolerized against dinitrofluorobenzene (DNFB). I.v. injection of cells into naive mice inhibited sensitization against DNFB in the recipients, but had no suppressive effect when injected i.v. into DNFB-immunized recipients. In contrast, injection of cells into the ears of DNFB-sensitized mice inhibited ear challenge with DNFB. Inhibition of challenge was hapten-specific since intracutaneous injection of DNFB-specific Ts into the ears of oxazolone (OXA)-sensitized mice did not affect challenge with OXA. When ears of OXA-sensitized mice were injected with DNFB-specific Ts and painted with DNFB before OXA challenge, CHS was suppressed. The inhibitory effect correlated with the local expression of interleukin-10 as shown by PCR. The data demonstrate that UV-induced Ts can suppress the effector phase of CHS provided they are present in the area of challenge. In addition, the activation of Ts is hapten-specific but once they are activated their suppressive activity is non-specific. This by-stander suppression seems to be mediated via the release of interleukin-10.

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The total amount of DNA damage determines ultraviolet radiation induced cytotoxicity after uniform or localized irradiation of human cells

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We have recently developed a micropore ultraviolet irradiation technique. An isopore membrane filter with 3 um diameter pores shields ultraviolet C radiation from cultured human fibroblasts, leading to partial irradiation within the cells with an average of about 3 exposed areas per nucleus. This study addressed the question of whether the spatial distribution of DNA damage within a cell nucleus is important in triggering ultraviolet-induced cytotoxicity. We have examined whether there are differences in cytotoxicity between partially ultraviolet-irradiated cells and uniformly irradiated cells after equal amounts of DNA damage were induced in the cell nuclei. We first determined DNA damage formation in normal human fibroblasts using an enzyme-linked immunosorbent assay. We found that 5 J per m2 ultraviolet irradiation produced an equivalent amount of cyclobutane pyrimidine dimers and (6-4) photoproducts per cell as 100 J per m2 with the membrane filter shield. At those doses, we found that both types of ultraviolet irradiation induced similar levels of cytotoxicity as assessed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Both types of ultraviolet-irradiated cells also had similar cell-cycle distribution and apoptosis as measured by flow cytometry. Moreover, no significant differences in repair kinetics for either type of photolesion were observed between the two different ultraviolet treatments. Similar results were obtained in Cockayne syndrome cells that are defective in transcription-coupled nucleotide excision repair. Present results indicate that in the range of photoproducts studied, the spatial distribution of DNA damage within a cell nucleus is less important than the amount of damage in triggering ultraviolet -induced cytotoxicity.

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Comparison of minimal erythema dose and minimal melanogenic dose induced by different wave-lengths of ultraviolet radiation

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The erythema and tanning responses of human skin to UV radiations which are used as diagnostic phototest and guideline of phototherapy, are different accordings to skin phototype and wave-lengths. Minimal erythema dose (MED), barely perceptible minimal melanogenic dose(MMDb) and well-defined border melanogenic dose(MMDw) of broad band UVA(BBUVA), UVA-1, broad band UVB(BBUVB), narrow band UVB(NBUVB) have not been reported. This study was done to measure the MED, MMDb and MMDw of different wave-lengths of UV radiation. Ten healthy volunteers were selected. the MED, MMDb and MMDw of the skin were assessed visually at 24 hours and 7 days after irradiation, respectively. The result were as follows: 1) The MMDb and MMDw induced by BBUVA and UVA-1 are less than MED. 2) The MED induced by BBUVB and NBUVB is less than MMDb and MMDw. 3) In BBUVB, the MED for photoskin type IV was the similar as the MMDb. Thease results suggest that the erythema and tanning response of the skin to UV radiations are different according to UV wave-lengths.

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Changes in cytokine network after UVB exposure on HaCaT, HKC-N6 and primo cultures

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Responses to UVB irradiation and grade of activation of keratinocyte cell line HaCaT, HKC-N6 and primo cultures were monitored by the production of cytokines (IL1β, IL6, IL8, IL10, IL12 and TNFα) and keratins K1/K10 and K5/K14. UVB doses 0.6; 0.8; 1.0; 1.5; 3.0; 5.0 and 10.0 mJ.cm-2 caused significant changes in cytokine production after 24h, 0.8 to 1.0 mJ.cm-2 particularly stimulated an increased production of IL6, IL8 and IL1β. Further TNFα was highly produced after 10.0 mJ.cm-2. Otherwise concentrations of total IL12 and NO production were not significantly changed. IL10 responsible for immune suppression after UVB exposure increased after 0.6 mJ.cm-2 or 0.8 mJ.cm-2, higher doses of UVB inhibited.its production. Production of the keratins K1/K10 and K5/K14 was monitored by RT-PCR. The only distinguishable keratin was K14 after 10.0 mJ.cm-2 in primo cultures. We can therefore see that UVB irradiation caused significant changes to the production of cytokines and keratins in the tested keratinocytes.

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Vitamin A exerts a photoprotective action in mouse and human skin by filtering UVB radiations

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Vitamin A is present in the skin, in particular the epidermis, mostly under retinyl esters, a storage form that can be converted into all endogenous forms of vitamin A. Retinyl esters absorb UV light between 300 and 350 nm, corresponding to a part of the sunlight received on the earth surface. We thus wondered whether retinyl palmitate - the predominant endogenous retinyl esters - was able to prevent biological actions of UVB due to its filter properties. We first used an in vitro model (Photochem. Photobiol. 75, 1-5 (2002)) to compare the photoprotective properties of retinyl palmitate to those of the sunscreen octylmethoxycinnamate, then we applied topical retinyl palmitate on the back of hairless mice, as well as on the buttocks of human volunteers, before exposing them to UVB. Thymine dimers, an index of DNA damage, were visualised in mouse epidermis and human punch biopsies by immunohistochemistry and quantified by immuno dot blots followed by densitometric analysis; the intensity of erythema 24 hours after UVB was analysed in human volunteers using a chromameter. In vitro, retinyl palmitate was shown to be as efficient as octylmethoxycinnamate in preventing UV-induced fluorescence or photobleaching of fluorescent markers, indicating a filter effect on UVB. In vivo, topical retinyl palmitate was as efficient as a sunscreen with SPF 20 in preventing both the formation of thymine dimers and the development of erythema in human skin exposed to 4 MED UVB, and significantly inhibited the formation of thymine dimers in mice exposed to 1 J/cm2 of UVB. Taken together, these results demonstrated that an endogenous form of epidermal vitamin A acts as a UV filter, and thus decreases the available UV energy that could destroy other epidermal biological molecules with deleterious consequences.

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Topical tacrolimus decreases UVB-induced DNA damage in mouse skin

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Squamous cell carcinomas on sun-exposed sites are a well known long-term complication in patients undergoing post-transplant immunosuppression with cyclosporin and/or tacrolimus (FK506). Recently, topical tacrolimus has been introduced for the treatment of atopic dermatitis. UV-induced cutaneous photocarcinogenesis is of theoretical concern in the setting of long-term treatment of sun-exposed skin sites in these patients. The aim of the present study was to investigate the in vivo effects of UVB on mouse skin treated with topical tacrolimus (Protopic 0.1%). Tacrolimus has an absorption peak at 290 nm, and a UV-filtering effect can be demonstrated using a liposomal model (Tran et al., Photochem. Photobiol. 75, 1-5 (2002)). After topical treatment once daily for 10 continuous days, hairless mice were exposed to 1 J/cm2 UVB, and sacrificed 1 or 24 hours after irradiation. Thymine dimers in epidermal DNA were visualised by immunohistochemistry, and quantified by immuno dot blots. Mice treated with either topical tacrolimus or vehicle but not exposed to UVB did not show any epidermal DNA damage. One hour following UVB-irradiation, vehicle-treated mice showed many positive nuclei for thymine dimers in histological slices, and immuno dot blots from epidermal DNA were strongly labeled for thymine dimers (pixel density of 100%). In contrast, mice treated with tacrolimus showed much less positive nuclei and the pixel density of immuno dot blots from epidermal DNA were only 16% of that of vehicle treated counterparts. These results suggest that 0.1% topical tacrolimus inhibits the formation of thymine dimers in UVB-exposed mouse skin, possibly via a UVB filter effect, for which tacrolimus has an absorption peak at 290 nm.

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Exceptionally high protection of photocarcinogenesis by topical treatment of green tea polyphenol (-)-epigallocatechin-3-gallate in hydrophilic ointment to SKH-1 hairless mice

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The use of botanical supplements with substantial antioxidant properties has received considerable interest to protect human skin from adverse effects of ultraviolet (UV) radiation. The green tea polyphenols are one of them. Here, we tested a cream based formulation of (-)-epigallocatechin-3-gallate (EGCG) in hydrophilic ointment (10%,w/w) for skin photoprotection in SKH-1 hairless mouse model. Topical treatment of EGCG (<1mg/cm2 skin area) before UVB irradiation (180mJ/cm2) - induced initiation and promotion stages of carcinogenesis protocol resulted in exceptionally high protection of photocarcinogenesis in terms of tumor incidence (70%), tumor multiplicity (82%) and tumor size (66%) in comparison to all previous studies conducted with EGCG. Treatment of EGCG also resulted in markedly high prevention of malignant conversion of papillomas into carcinomas. The treatment of EGCG also resulted in prevention of multiple UVB irradiation (90mJ/cm2 on alternate days for one month)-induced depletion of glutathione peroxidase (78%), catalase (89%) and glutathione level (85%) concomitant with reduction in UVB-induced lipid peroxidation (76%) and protein oxidation (67%) in the skin. Because expression of matrix metalloproteinases (MMPs) have been shown to be associated with the invasion and metastasis potential of skin cancer, in additional experiments using gelatin zymography and Western blotting, treatment of EGCG resulted in marked inhibition of multiple UVB irradiation-induced expression of MMP-3 (47%), MMP-7 (88%) and MMP-9 (86%). Together, for the first time a cream based formulation of EGCG was tested to explore the possibility of its maximum photoprotective capability, and the data from this study suggest that EGCG could be useful to attenuate solar UVB radiation-induced risk of nonmelanoma skin cancer and other skin disorders in humans.

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Different effects of PUVA and narrow-band UVB on human melanocyte regeneration

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Both psoralen plus UVA (PUVA) and narrow-band (311nm) UVB radiation are effective treatments for vitiligo vulgaris. However, the mechanisms for PUVA and narrow-band UVB in repigmentation are not thoroughly clarified. Narrow-band UVB irradiation stimulated a significant increase in human cultured melanocytes migration. Nevertheless, PUVA demonstrated no significantly stimulatory effect on human melanocytes migration. Both treatments could stimulate a significant increase in interleukin 8 (IL-8) release from human melanocytes. The expression of phosphorylated $p125^{FAK}$ on narrow-band UVB-irradiated melanocytes were more prominent as compared to PUVA-treated melanocytes. Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) activity in supernatants by narrow-band UVB-irradiated melanocytes and keratinocytes were significantly increased as compared to PUVA-treated group. Supernatant derived from narrow-band UVB-irradiated keratinocytes, but not PUVA-treated keratinocytes, could stimulate the proliferation of human melanocytes. Moreover, narrow-band UVB induced a significant increase in basic fibroblast growth factor (bFGF) and endothelin 1 (ET-1) release from keratinocytes.These results suggested that the mechanisms of PUVA and narrow-band UVB on melanocyte regeneration were different.

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Induction of UVB-induced tolerance does not only induce T regulatory cells but also T effector cells

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Ultraviolet B light (UVB) induces tolerance which is due to the induction of hapten-specific T regulatory cells (Treg). Since sensitization results in the induction of both T effector cells and Treg we asked whether induction of tolerance by UVB does not only result in the induction of Treg but also of T effector cells. C3H/HeN mice were first tolerized by painting dinitrofluorobenzene (DNFB) onto UVB-exposed skin. In addition, we utilized a soluble form of the protein Dectin-2 (sDec2). Dectin-2 is a co-stimulatory molecule expressed selectively on dendritic cells which appears to play a pivotal role in UVB-induced tolerance. sDec2 binds to UVB-induced Treg since depletion of sDec2-bound T cells obtained from UVB-tolerized mice results in the loss of transfer of suppression. FACS analysis of T cells obtained from UVB-tolerized mice revealed that sDec2 only bound to $CD4^+$T cells. sDec2-bound $CD4^+$T cells were mainly positive for CD25, a phenotype similar to Treg. $CD4^+$ T cells from UVB-tolerized mice were further fractionated into sDec2-bound T cells and injected *i.v.* into naive syngenic mice. Recipients receiving sDec2-bound T cells could not be sensitized against DNFB, indicating that sDec2-bound, $CD4^+CD25^+$ T cells transfer UVB-induced tolerance. In contrast, recipients receiving sDec2-unbound T cells could be normally sensitized. Even more importantly, a specific ear swelling response could be induced in the recipients of sDec2-unbound T cells when the animals were not sensitized but only challenged with DNFB. This indicates that the fraction of sDec2-unbound T cells obtained from UVB-tolerized mice must contain DNFB-specific T effector cells. Together, these data indicate that induction of tolerance by UVB does not only induce Treg but also causes the induction of T effector cells.

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EGF receptor activation by UV irradiation is mediated via HB-EGF shedding in human keratinocytes

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UV irradiation has various biological effects on skin. One is EGF receptor (EGFR) phosphorylation and the subsequent activation of MAP kinase, including JNK and ERK. However, the cross-talk mechanism linking UV irradiation and EGFR phosphorylation remains unknown. Recently, it was reported that EGFR transactivation by G-coupled-protein receptors and their ligands is mediated via HB-EGF shedding, the conversion of HB-EGF from a membrane-anchored form to a soluble form. This led us to hypothesize that UV irradiation-induced EGFR phosphorylation is mediated via HB-EGF shedding. First, we examined the effect of UV irradiation on EGFR phosphorylation in human keratinocytes. UV irradiation (30 mJ/cm^2) induced EGFR phosphorylation in a time-dependent manner, optimally 2.1-fold at 20 min. Next, we studied whether UV irradiation is able to induce HB-EGF shedding from the keratinocyte cell surface. Biotinylated membrane-anchored HB-EGF on the keratinocyte cell surface disappeared almost completely 10 min after UV irradiation. The soluble form of EGFR ligands in culture medium was measured by bioassay using the EP170.7 cell line, which is a stable transformant expressing EGFR. Soluble EGFR ligands in culture medium increased optimally 1.5-fold at 5 min after UV irradiation. An inhibition assay using anti-HB-EGF blocking antibody identified 77% of the soluble EGFR ligands as HB-EGF. Furthermore, KB-R8301, a specific inhibitor of HB-EGF shedding, inhibited the UV irradiation-induced HB-EGF shedding almost completely at 1 μM. Finally, we studied the effect of KB-R8301 (1 μM) and anti-HB-EGF blocking antibody (10 μg/ml) on EGFR phosphorylation induced by UV irradiation. Both inhibited UV irradiation-induced EGFR phosphorylation almost completely. Combined, these data demonstrate that UV irradiation-induced EGFR phosphorylation is mediated via HB-EGF shedding, the conversion of HB-EGF from a membrane-anchored form to a soluble form.

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Studies of 8-MOP/UVA influence on formation of skin photoaging

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There are more and more evidences that longwave ultraviolet (UV) radiation (UVA; 320-400 nm) plays an important role in the pathogenesis of photoaging and large-scale mtDNA deletions in UVA radiation-induced is believed to be involved in.But there is lack of a reliable and practical human model for research of its possible mechanisms yet. Appearance of premature aging of the skin is the prominent side effect of photochemotherapy with psoralnes and ultraviolet A (PUVA) in dermatology,it is possible that the combined treatment of 8-MOP and UVA would provide models in vivo and in vitro for study of skin photoaging.In our studies, the skin photoaging-characteristic biological markers such as histopathology of skin dermis; expression of SA-beta-galactosidase; expression of p16 protein; expression of matrix metalloproteinases (MMPs) and their tissue inhibitor of matrix metalloproteinases(TIMP-1); accumulations of mutations of mtDNA, are observed in biopsies of the non-lesion back skin of vulgar psoriatic patients who had been treated by PUVA and the cultured foetus dermal fibroblasts combined treatement with 8-MOP and UVA. With combined treatment of 8-MOP and UVA, the photoaging-characteristic biological markers of the dermal fibroblasts in vivo and in vitro were changed as follows: 1. with a permanent switch of mitotic to stably postmitotic phentypes, fibroblasts displayed growth suppression and morphological changes of cell senescence; 2. increased expression of SA-beta-galactosidase; 3. increased expression of p16 protein; 4. continuous up-regulation of mRNA of MMP-1 and MMP-3,while the protein of TIMP-1 is only slightly induced; 5. high levels of the 4977bp deleted mtDNA accumulated in dermis and cultured fibroblasts.These results suggested that the in vivo and in vitro models may serve as useful tools for the research of the mechanisms of photoaging,and also may serve as useful tools for research of the mechanisms of mutations of mtDNA in skin photoaging.

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Photoaged skin showed lower ERK and higher JNK/p38 activities than intrinsically aged skin of the same individuals

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Many of the molecular alterations resulting from chronological aging and exposure to UV irradiation are believed to arise largely from oxidative stress. Previously, we have demonstrated decreased extracellular-signal-regulated kinase (ERK) and increased stress-activated MAP kinase activities (JNK and p38) in intrinsically aged human skin in vivo, compared with young skin. In this study, MAP kinase signaling between photoaged and intrinsically aged skin of the same individuals was compared. Interestingly, ERK activity in the photoaged skin was decreased more, and activities of JNK and p38 were increased more than intrinsically aged skin of the same individuals, respectively. c-Jun expression and AP-1 activity in photoaged skin tended to be higher than intrinsically aged skin of the same individuals. We also demonstrated that catalase activity in the dermal fibroblasts was decreased with skin aging, and H2O2 was accumulated in aged human dermal fibroblasts. Treatment of catalase reversed the aging-dependent changes of MAP kinases in cultured aged fibroblasts. Catalase increased ERK activity and inhibited JNK and p38 activities in aged fibroblasts. Our results suggest that the changes of MAP kinase pathways resulting from skin aging and photoaging may arise largely from accumulation of reactive oxygen species due to lower level of catalase. This central role of oxidative stress may explain in part the observed similarity of signaling changes involved in photoaging and chronological skin aging.

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***Polypodium leucotomos* extract (Fernblock) inhibits photoisomerization and photodescomposition of trans-urocanic acid**

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Trans-urocanic acid (t-UCA) is an epidermic pigment derivated from histidine endowed with photoprottective and antiradicalary properties due to the imidazole moiety. UVB induces photoisomerization to cis-UCA (probably to which skin immunosuppression is due); moreover, when UVB acts in presence of H202 or free radicals, photodescomposition is produced. To the breakdown products are also immunosupression activities atributed. It is to remark that in the presence of solar irradiation (UVA+UVB) and a photosensitizer, UVB+ROS acting together could generate either isomerization or descomposition of trans-urocanic acid. Polypodium leucotomos extract (Fernblock) has shown in several assays its antioxidative and immunoprotective activity. In this work, we study the effect of fernblock on tUCA when irradiated with UVB in absence or presence of oxidative agents. t-UCA was irradiated by UVB light and photoisomerization to cis-UCA was monitorized by reverse phase HPLC (RP-HPLC). When irradiated in the presence of H202 the breakdown profile was also analyzed by RP-HPLC. In both cases a very clear protective activity was found. We are showing a very simple in vitro model, which allows to demonstrate that Fernblock protects t-UCA from UVB irradiation effects ever in oxidative conditions. These properties may be of importance to its photoimmunoprotective activity.

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Flashlamp pumped dye laser did not cure papulo-pustolar rosacea

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Patients with both the erythematotelangiectatic and the papulopustular type of rosacea were treated with FPDL until telangiectases disappeared. The aim of the study was to see if the treatment result might be effective on the papules and the pustules. Ten women were treated (mean age 45 years, range 33-74) on one side of the face with FPDL (wavelength 585 nm, spot size 5 mm, pulse duration 0.45 ms) in average 2,4 treatments (range 1-4). Before treatment each side of the face was estimated with regard to erythema, telangiectases, papules, pustules or swelling with a scale 0-3 (0=absent, 1=mild, 2=moderate, 3=severe). The final evaluation was performed blind in average 10 months (range 3-28) after the last treatment with a scale 1-5 (1=worse, 2=unchanged, 3=slightly improved, 4=much improved, 5=cured/no efflorescenses). No other treatment was allowed during the treatment and follow-up period. Two of the patients had significantly less papulopustular lesions after treatment, three had slightly less, three were unchanged and two had more lesions. Our conclusion from this rather small sample of patients is that FPDL has a good effect on erythematotelangiectatic rosacea, but limited value on papulopustular lesion. This means that the origin to rosacea is probably not only vascular.

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Anti-pruritic mechanism of PUVA therapy to patients with atopic dermatitis

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There is a recent increase in the number of patients with atopic dermatits (AD),especially severe and refractory cases to the standerd therapies for AD such as steroid ointment. Psoralen photochemotherapy with UVA (PUVA) has been reported to be successfully substitutional for,or an adjunct to,conventional treatment in AD patients. We examined effects of PUVA on cutaneous neurologic factors,especially on substance P (SP) and neurokinin receptor (NK-1R),in AD. Topical PUVA therapy for 12 AD patients resulted in the rapid improvement of disease severity and of itch intensity. Immunohistochemical study revealed decreases in intraepidermal innervation of NK-1R on vascular endothelial cells as well as on keratinocytes in AD lesional skin. Densitometrical analysis of NK-1R expression on endothelial cells demonstrated approximaterly 75% reduction of endothelial-expression of NK-1R and total UVA dose-dependent reduction of NK-1R immunoreactivity. Ultrastructurally,degenerative changes of nerve endings were observed just beneath the epidermis. Although mast cells in AD lesional skin possess a larger amount of SP within their specific granules compared with the controls,after PUVA therapy,there were significant decrease in SP-containing mast cell granules and in SP-binding gold particles per a granule at the immunoelectron microscopic level. In vitro studies using cultured human neural cells showed cell damage represented by disappearance of neurites in neural cells and disappearance of NK-1 mRNA expression in vascular endothelial cells. These results suggest that a variety of anti-pruritic mechanisms may lead to successfultherapeutic efficacy of PUVA for AD,which include decrease in the density of SP-containing nerve fibers and in the amount of SP within mast cells,degenrrationof nerves,inhibition of mast cells degranulation,the modulation of NK-1R,and so on,which may in turn contribute to a marked relief of itch intensity in severe cases of AD.

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Artemia extract significantly down-regulates ATRA-induced IL-1 after UV exposure in human fibroblasts

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IL-1 is an important pro-inflammatory cytokine that exhibits pleiotropic activities. IL-1 is synthesized by a large number of cells, including skin cells, and exists under two forms, IL-1 alpha, and beta. Retinoic acid and its derivatives have been widely used in topical skin care products for therapeutic and cosmetic purposes. Our prior studies have shown that Artemia extract (AE) administration to cells treated with ATRA for a short time, down-regulated IL-1 expression in these cells. As ATRA-treated skin is known to be fragile toward UV stress, we were therefore interested in investigating the effect of AE on long-term ATRA-treated cells, with and without UV stress. Intra IL-1 beta level was assessed in the following ELISA studies on cultured human fibroblasts treated with ATRA 100 nM, for a long time (up to 2 months), with and without UV irradiation with 100 mJ/cm2. ELISA was performed at different time points during the test period and the results were submitted to statistical analysis using the Mann-Whitney test which compares the AUC scores under different conditions, and also compares the findings at different time points. Our results show that in UV-irradiated cells (AUC +46%), AE significantly reduced IL-1 beta at different time points in the study (AUC -60%), confirming that AE down-regulates UV-induced inflammatory cytokines. Furthermore, cells treated with ATRA and irradiated with UV exhibited a consistently higher level of IL-1 than control cells (+22.35% increase of AUC over control UV). Interestingly, AE administration to ATRA-treated cells significantly decreased UV-induced IL-1 in the cells at different time points in the study (AUC -44%). Moreover, this effect of AE on ATRA-treated and irradiated cells was remarkable and IL-1 level reached lower levels than in the UV control cells. These results suggest a very interesting application for Artemia extract, in association with long-term Retinoids therapy of the skin, in increasing skin comfort especially toward UV stress

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***Polypodium leucotomos* extract (Fernblock) antagonizes ultraviolet-induced free radicals hyperproduction produced in presence of titanium dioxide**

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Titanium dioxide (TiO2) is a photocatalitic reactive due to its semiconductor condition, with electronic transition promoted by UVA light (photoexcitation). Despite this fact, it is widely used in sunscreen formulations. It is possible to evaluate its photocatalytic capacity by using different biological models: non-cellular (enzymes) or cellular models. The aim of this work is to show how a Polypodium leucotomos extract (Fernblock), an antioxidative and photoprotective natural extract is able to protect either enzymes or cellular system against UVA/TiO2 induced damage. We used as enzymatic system horseradish peroxidase. Photolysis was induced by UVA irradiation in presence of TiO2. As a consequence of oxidative damage, the enzyme denatured. This enzyme denaturation was evaluated by measuring enzymatic activity using H202 and ABTS as sustrate. Additionally, human erytrocytes and fibroblast cells were used as cellular system and UVA irradiation was performed in presence or absence of TiO2 or Fernblock. Damage in human red cells was evaluated by hemolysis, monitorized at 540 nm; in the case of fibroblasts by analyzing survival by the MTT method. As expected TiO2 potentiates UVA induced damage in enzymatic or cellular system. Fernblock is able to antagonize the effect of the UVA and moreover the effect the UVA+TiO2: preservation of enzymatic activity, reduction of hemolysis (red cells) and survival (fibroblasts) up to 100%. In conclusion, Fernblock significantly minimize the hyperproduction of free radicals induced by UVA in presence of TiO2. This antiradicalary property is demonstrated in cellular and non-cellular system. Therefore, Fernblock may be a excellent adjunct to TiO2 in sunscreen formulations.

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PUVA arrests cell cycle progression exclusively in S-phase by activating a caffeine sensitive DNA damage checkpoint

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Induction of interstrand crosslinks (ICLs) in chromosomal DNA is considered a major reason for the anti-proliferative effect of psoralen plus ultraviolet A (PUVA). Our understanding of how these ICLs inhibit cell proliferation is incomplete. In order to maintain genomic stability, cells halt cell cycle progression at certain checkpoints in response to DNA damage to prevent replication of damaged DNA templates or segregation of broken or incompletely replicated chromosomes. Here we show that HaCaT keratinocytes treated with PUVA arrest with S-phase DNA content. Cells that had completed DNA replication (G2 cells) were not perturbed by PUVA and passed through mitosis. Likewise, cells PUVA treated in G1 progressed normally until initiating DNA replication. The replication arrest was overridden by caffeine, a known inhibitor of the ATM/ATR checkpoint kinases. PUVA induced rapid phosphorylation of the ATM substrate Chk1 at Ser345 and a concomitant decrease in Cdc25A levels. Phosphorylation of Chk1 and the decrease of Cdc25A levels were both abolished by caffeine. Our results suggest that PUVA induced ICLs are sensed as a relevant DNA damage only during ongoing replication (S-phase). The sensitivity to caffeine further suggests that active checkpoint signaling rather than mechanical blockage of replication forks by ICLs causes the PUVA induced growth arrest.

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The *in vitro* effect of UVB on nitric oxide production in human telomerase immortalized keratinocytes

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Nitric oxide has already been proven to be a critical biological mediator with many physiologic and patho-physiologic functions. The role in inflammation has important dermatologic implications but the exact mechanism of UVB induction of nitric oxide has not yet been elucidated. Both eNOS and iNOS have been immunohistochemically localized in keratinocytes. The purpose of this study was to determine what effect increasing doses of UVB irradiation had on nitric oxide production in human telomerase immortalized keratinocytes (hTERT). Futhermore, we intended to determine any relationship between eNOS and nitric oxide production. In the first part of the experiment hTERT cells were irradiated with UVB at doses ranging from 0-100 mJ/cm2. Cell associated and supernatant nitric oxide was measured with Griess assay. In the second part of the experiment hTERT cells were irradiated with a similar range of UVB doses and eNOS mRNA was measured with ELISA at 12, 24, and 48 hours after stimulation. Our results showed that UVB induced nitric oxide production at all doses over non-treated cells. At 12 hours the quantity of eNOS mRNA was greater than baseline with all doses of UVB. At 24 hours there was no significant increase of eNOS mRNA over non-treated cells at any dose of UVB. At 48 hours eNOS mRNA was increased again over baseline at all doses of UVB. In conclusion, these results show that UVB does play a role in nitric oxide production in hTERT keratinocytes and although eNOS is a constitutive enzyme, UVB at varying doses appears to affect the amount of eNOS mRNA that is present. There seems to be a biphasic upregulation of eNOS mRNA, increasing at 12 and then 48 hours. Consequently, eNOS might be responsible for an increase in nitric oxide during UVB stimulation during both short and long periods after exposure and ultimately play an important role in inflammation.

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Dietary feeding of procyanidins from grape seeds prevents photocarcinogenesis in mouse skin: prevention of photocarcinogenesis associated with the inhibition of ultraviolet radiation-induced oxidative stress and cellular signaling

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There is considerable interest in the use of dietary botanical supplements with substantial antioxidant activity as a complementary and alternative medicine to protect skin from the adverse biological effects of solar ultraviolet (UV) radiation. Procyanidins isolated from grape seeds (GSP) have been shown to have antioxidant properties. In the present study, we tested whether dietary feeding of GSP to SKH-1 hairless mice prevented photocarcinogenesis by employing long-term animal tumor protocol concomitant with the mechanism of protection by GSP treatment. Dietary feeding of GSP (0.2% and 0.5%, w/w) when administered during a UVB (180mJ/cm2)-induced multistage carcinogenesis protocol prevented skin tumorigenesis in terms of tumor incidence (20-30%), tumor multiplicity (45-57%) and tumor size (56-75%). Feeding of GSP (0.5%, w/w) also inhibited malignant conversion of papillomas to squamous cells carcinomas concomitant with reduction in carcinoma incidence (45%), carcinoma multiplicity (67%) and size of the carcinoma (79%). Dietary feeding of GSP also prevented chronic UV exposures-induced depletion of antioxidant defense enzymes such as glutathione peroxidase (202%), catalase (81%) and glutathione content (43%), and inhibited UVB-induced lipid peroxidation by 70%. Further, inhibition of UVB-induced oxidative stress by GSP was delineated with cellular signaling of mitogen-activated protein kinase (MAPK) pathways. Western blot analysis revealed that dietary intake of GSP (0.5%, w/w) inhibited UVB radiation-induced phosphorylation of ERK1/2 (45%), JNK (58%) and p38 (86%) proteins of MAPK family. The results of this study provide evidence that GSP possesses anti-photocarcinogenic effects, which may be associated with the prevention of UVB-induced oxidative stress and/or diminution of activation of cellular signaling cascade of MAPK pathways in vivo mouse skin.

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Reinforcing properties of UV light in frequent indoor tanners

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Use of tanning beds is a widespread behavior that is injurious to skin. The purpose of this study was to assess whether tanning has reinforcing properties that contribute to the behavior. Fourteen frequent tanners were enrolled in a controlled, single-blind, repeated-choice trial of UV exposure for a six-week study period. Subjects underwent UV and non-UV exposures in a commercial tanning apparatus on Monday and Wednesday. Subjects could not discriminate the UV/non-UV filters on the basis of appearance. After the exposures on Wednesday, their bed preference was recorded. On Friday, they were given the opportunity to choose to tan in either (or both) of the two beds. 13 of 14 subjects consistently preferred the UV exposure to the non-UV exposure stimulus. In 12 subjects that chose additional tanning exposure, 11 consistently chose the UV bed for that exposure. The clear behavioral choice of UV stimulus was mirrored by the reported preference for the UV bed. Greater "relaxation" was reported after UV exposure compared to after non-UV exposure ($p<0.05$). These data support a UV induced mechanism that alters mood and sensation in frequent tanners. This reinforcing phenomenon may be an important contributor to tanning behavior in frequent tanners.

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Efficacy of topical white tea against UV-induced Langerhans cell depletion and DNA damage in human skin

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There is growing interest in the cutaneous photoprotective effects of antioxidants. Previous studies show that topical green tea polyphenols decrease damage induced by UVR. White tea, the least processed of all tea formulations, has not been investigated for photoprotective effects. We aimed to determine the effects of four compounds: 0.5% green tea polyphenol, 2% white tea extract, 0.2% white tea powder, and 3% bioconverted white tea, on solar-simulated radiation (SSR)-induced erythema, depletion of CD1a+ Langerhans cells, thymidine dimer formation, and oxidative DNA damage via 8-OHdG levels, in normal human skin. The compounds were applied over gluteal skin in 10 volunteers, 15 minutes before and immediately after 2 MED of SSR exposure. Colorimetric evaluation of erythema was performed and punch biopsies obtained for immunohistochemistry. All four compounds decreased erythema, although green tea alone resulted in statistically significant reduction, relative to untreated unirradiated skin. All tea formulations resulted in significant reversal of Langerhans cell depletion (32-61%), as well as reduction of thymidine dimer (33-69%) and 8-OHdG (28-47%) levels. These results confirm the photoprotective benefits of green tea polyphenols and demonstrate for the first time the comparable efficacy of more cosmetically acceptable white tea formulations in preventing UV-induced damages.

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Xeroderma pigmentosum group A gene-deficient, stem cell factor-transgenic mice with epidermal melanin are resistant to UV-induced immunosuppression and carcinogenesis

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Xeroderma pigmentosum group A gene-deficient (XPA(-/-)) mouse is a useful model of XP. However, they have no melanin and melanocytes in the interfollicular epidermis. In the present study, we developed XPA (-/-), stem cell factor transgenic (SCF(+/-)) mice, which have a defect in nucleotide excision repair and have epidermal melanocytes and melanin. Using this animal, we investigated the protective effects of epidermal melanin on the cutaneous changes induced by DNA injuries. XPA(-/-), SCF(-/-) mice showed a significant ear swelling response (ESR) after UVB radiation or DMBA application, by which DNA damage is not repaired in XPA(-/-) mice. However, XPA(-/-), SCF(+/-) mice developed ESR by DMBA but not by UVB. The induction of apoptotic cells and cyclobutane pyrimidine dimmers (CPD) by UVB radiation were also highly suppressed in XPA(-/-), SCF(+/-) mice. Contact hypersensitivity (CHS) was not inhibited in XPA(-/-), SCF(+/-) mice. On the other hand, when mice were treated with DMBA, there were no significant differences in ESR, apoptotic cell induction, CPD formation, and immunosuppression of CHS between XPA(-/-), SCF(-/-) and XPA(-/-), SCF(+/-) mice. In XPA(-/-), SCF(-/-) mice, skin tumors were easily induced by UVB radiation at a total dose of 1J/cm2 or DMBA application. However, in XPA(-/-), SCF(+/-) mice, skin tumors did not develop even with a total dose of 72J/cm2 of UVB radiation. In contrast, DMBA treatment induced skin tumors in XPA(-/-), SCF(+/-) mice as well as XPA(-/-), SCF(-/-) mice. These findings indicate that epidermal melanin has a high ability to protect DNA damages by UVB radiation, and thereby, prevents UV-induced inflammation, immunosuppression and carcinogenesis. These mice are a useful animal to investigate a role of melanin in UV-protection, since they have melanocytes in the epidermis similarly to human.

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Spatial confinement of 5-aminolevulinic acid-based photodynamic therapy by thermal and chemical inhibition

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Topical application of aminolevulinic acid (ALA) results in the accumulation of protoporphyrin IX (PpIX) in the epidermis and in cutaneous adnexal structures, including hair follicles and sebaceous glands. ALA-photodynamic therapy (PDT) has been shown in preliminary studies to be an effective modality for hair removal and for the treatment of acne. However, PDT-induced side effects in the epidermis—such as superficial exfoliation, crusting, and hyperpigmentation—limit clinical applications. To improve current methods for PDT, we explored the spatial confinement of ALA-induced PpIX formation by both thermal and chemical inhibition. PpIX formation was demonstrated to increase quasi-exponentially with temperatures between 15 and 40 C in a rat epidermal keratinocyte (REK) cell line. Incubation of REK cells with 0.25 mM ALA at 25 C for 3 hours compared to incubation at 37 C resulted in a 5-fold (80%) decrease in PpIX formation ($p < 0.001$); incubation at 20 C compared to 37 C resulted in a 17-fold (94%) decrease ($p < 0.001$). Chemical inhibition was achieved by co-exposure of REK cells to ALA and 4,6-dioxoheptanoic acid (DOHA); greater than 90% inhibition of PpIX formation occurred with 1 μM DOHA. Both thermal and chemical inhibition strategies were successfully employed on human skin. Topical application of a 5% ALA solution for four hours resulted in epidermal fluorescence that could be focally suppressed both by local contact cooling of the epidermis to 17 C and by local application of a 0.3% DOHA solution. These results provide promising evidence for two approaches to spatially confine the formation of PpIX that may lead to improved methods for PDT. By suppressing the formation of PpIX in the epidermis, PDT-induced side effects may be diminished sufficiently while allowing deeper-lying targets to receive a therapeutic PDT dose. Practical applications of these techniques include PDT for hair removal and for the treatment of acne.

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Lomefloxacin enhances UVA-induced skin tumors in xeroderma pigmentosum group A gene-deficient mice

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Lomefloxacin, a fluoroquinolone antibacterial agent,is phototoxic and phototumorigenic in mouse skin. The mechanisms of phototumorigenesis of quinolone drugs have not been fully elucidated,while those of phototoxic reaction have been well studied.Formation of cyclobutane pyrimidine dimers(CPD) by UVB radiation is primarily involved in carcinogenesis. On the other hand,UVA region is mainly responsible to photobiologic reactions of quinolone drugs. Then, we investigated phototumorigenesis of lomefloxacin using xeroderma pigmentosum group A gene-deficient (XPA(-/-))mouse,which is defective in nucleotide excision repair. XPA(-/-)mice and XPA(+/+) mice were irradiated to dose of 5J/cm2-UVAthree times a week with or without lomefloxacin.In XPA(-/-),the first skin tumor appeared after 5 weeks. The majority of skin tumors were squamous cell carcinomas.In XPA(+/+),the first tumor appeared after 23 weeks.The number of tumors developed was much more in XPA(-/-) than in XPA(+/+) mice. Immunohistochemically,CPD formation was obserbed after UVA-exposure in skin of XPA(+/+) and XPA(-/-) mice which had been given lomefloxacin. However, the CPD disappeared earlier from XPA(+/+) mice than and XPA(-/-) mice.These results indicate that lomefloxacin and subsequent UVA exposure induces DNA damage,and exerts its phototumorigenesis.

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Ultraviolet B radiation mediates nuclear translocation of the aryl hydrocarbon receptor

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The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor/nuclear receptor that mediates toxicity of various exogenous chemicals like Dioxin and polycyclic aromatic hydrocarbons by translocation into the nucleus. There it dimerizes with its partner ARNT before it binds to specific DNA recognition sequences (XREs) and subsequently activates genes of the AhR gene battery. This gene battery includes genes that are involved in drug metabolism (e.g. cytochrome P450) as well as xenobiotic metabolism unrelated genes (e.g. TGF-alpha, IL-1beta). UV B radiation is a well know modulator of the expression of several of these genes in human keratinocytes and an induction of CYP dependent monooxgenases has been demonstrated in UVB-irradiated human skin. In the present study we have tested the hypothesis that the AhR is a molecular target for UVB. Accordingly, we transiently transfected cells from the spontaneously immortalized keratinocyte line HaCat with a green fluorescent protein (GFP) coupled AhR. Fluorescence microscopy revealed that in these cells, translocation of the GFP-AhR protein from the cytoplasm into the nucleus could be observed upon stimulation with the AhR ligand Benzo(a)pyrene which served as a positive control. In contrast, no nuclear translocation was observed if cells were sham-irradiated or exposed to UVA (340-400 nm) radiation at dose levels (30 J/cm2) which were sufficient to induce CYP1A1 mRNA expression. In contrast to UVA radiation, UVB irradiation (10 mJ/cm2) shuttled the AhR into the nucleus in a time dependent. This nuclear translocation was associated with a 2-fold upregulation of CYP1A1 mRNA steady state levels 6 hours after UVB radiation exposure. These studies demonstrate that UVB radiation affects AhR signaling in human keratinocytes. To the best of our knowledge this is the 1st report indicating that UVBR has the capacity to induce the translocation of a nuclear receptor in mammalian cells. Ongoing studies are directed at the identification of the putative physiologic ligand of the AhR in UVB-irradiated keratinocytes.

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Effects of ultraviolet radiation on hairless rats: parameters related to oxygen free radicals and Langerhans cells

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Using the hairless rat model (Romeu et al., Life Sciences, 2002), we studied the effects of UV radiation (UVR) on 1) parameters related to oxygen free radicals (OFR) in epidermis and blood and 2) on Langerhans cells (LC). Two groups of rats (n=7 each) received two UV doses (2.14 J/cm2 at an interval of 24h; UVB/UVA =0.83) on half of their dorsal skin while the other half was protected. Animals were sacrificed at 72h and at 120h after the second dose of UV. A third group (C) was sham irradiated. In erythrocytes and epidermis we determined: catalase (CAT), glutathione peroxidase (GPx), reduced (GSH) and oxidized (GSSG) glutathione, GSH reductase (GR), GSH transferase (GST), superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBARS). GSH, GSSG and TBARS were also analyzed in plasma. In comparison with group C, after UVR, GSH decreased by 60% at 72h and GSSG decreased by 54% at 72h and 120h in irradiated epidermis; in erythrocytes, GST increased by 49% and GPX decreased by 26% at both 72h and 120h, and CAT increased by as much as 35% at 72h and was partially restored at 120h. In irradiated epidermis, GSSG, GSH and the GSH/GSSG ratio were lower (20%, 37% and 21% respectively) at 72h whereas CAT was higher (21%) than in the non-irradiated epidermis of the same animal. ATP-ase positive LC decreased significantly (p=0.010) in irradiated epidermis in comparison with non-irradiated epidermis (21% at 72h and 17% at 120h). Multiple linear regression analysis showed that epidermal GSSG, GR and GPx were good predictors of the LC number. In an additional study, rats pretreated with a sunscreen (SPF around 15) with active anti-OFR ingredients received a total UVR dose equivalent to 15 times the minimal erythemal dose. Despite a slight erythema, the number of LC did not decrease in comparison with control rats. This suggests that erythema and the immunosuppresive effects of UVR can be prevented independently.

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Experimental photopheresis induces cells with regulatory/suppressor activity

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The basis of extracorporeal photopheresis is the reinfusion of leukocytes, which have been exposed to 8-methoxypsoralen (8MOP) and UVA. Besides its beneficial effect in cutaneous T cell lymphoma, photopheresis has shown evidence of benefit for the treatment of autoimmune diseases, solid organ transplant rejection, GvHD and atopic dermatitis. The underlying mechanism for this effect remains unresolved. Since UV radiation of the skin exhibits the capacity to induce tolerance (in this case via regulatory T cells), we asked whether photopheresis might induce a state of tolerance following extracorporeal treatment. For this study we utilized a murine model of contact hypersensitivity. Splenocytes and lymph node cells which were sensitized with dinitrofluorobenzene (DNFB) were exposed to 8MOP plus UVA in vitro. 8MOP/UVA-treated cells were injected i.v. into naive mice which were subsequently skin sensitized with DNFB and ear thickening measured following ear challenge. Animals which had received 8MOP/UVA-treated cells were significantly suppressed in their contact hypersensitivity (CHS) response. In contrast, mice which received cells which were untreated or exposed to UVA or 8MOP alone were not suppressed in their CHS response. This suppression was cell-mediated and antigen specific as demonstrated by the ability to transfer the tolerance to a naive animal which could, however, properly respond to the unrelated hapten oxazalone. Taken together, these data suggest that infusion of 8MOP/UVA-treated cells from sensitized mice induces a state of tolerance which is antigen-specific and cell mediated. Additional studies are underway to determine which type of regulatory T cells mediate this effect.

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Repetitive UV irradiation of cultured skin cells to generate the mitochondrial DNA common deletion

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We have shown that an increased incidence of the 4977bp common mitochondrial DNA (mtDNA) deletion has been strongly associated with increased ultraviolet radiation (UVR) exposure in human skin. A single study has also shown that the common deletion (CD) can be generated through repetitive doses of UVA in cultured skin fibroblasts. The aims of our study were 1) to induce the CD in cultured skin cells using a UV light source with an output closer to that found in sunlight; 2) to provide an acccurate and reliable means of measuring low-levels of the CD generated in the cells by UVR. Using a UVA+UVB light source (Helarium 40W lamp (Wolff B1.01), 290-400nm, peak emisssion at 325nm), we tested a whole series of repetitive UV dose regimes to generate the CD in a cultured HaCaT cell line. The optimal strategy used an alternative daily dose of 500mJ/cm^2, which generated the CD over a period of approximately 35-40 days. The levels of the CD were too low for accurate determination by our previously used 3-primer PCR assay. Therefore, we compared an established and reliable, but time consuming, serial dilution PCR method for measuring the CD, with a nested PCR strategy that was relatively simple to perform as detailed in a previous in vitro study using UVA (Berneburg *et al., J Biol Chem,* **274,** 15345-9, 1999). Using the serial dilution PCR method, the first detectable sign of the CD occurred after 17 repetitive doses of 529 mJ/cm^2 UVR. Two further UVR doses caused a 500-fold increase in the level of the CD. In contrast, the nested PCR technique failed to detect any differences in the levels of the CD from the same DNA samples and should not be used for accurate determination of the CD. This is important because once a certain threshold of damage is achieved, it will impair mitochondrial respiratory function, thereby affecting skin biology through the induction of apoptosis and reactive oxygen species.

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The platelet-activating factor pathway is involved in psoralen+UVA (PUVA)-induced skin damage and apoptosis

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PUVA photochemotherapy is one of the most effective treatment modalities for a large variety of skin diseases. However, long-term exposure to PUVA (as often needed in psoriasis) has serious side effects, such as the formation of skin cancers and promoted skin aging. The exact molecular mechanisms by which PUVA results in acute and chronic skin damage are not exactly defined, however, we hypothesized that the proinflammatory PAF pathway (Walterscheid et al, J Exp Med 195, 2002) may be involved. We investigated whether the intervention in the PAF pathway by application of a specific PAF-receptor antagonist or a selective COX-2 inhibitor can abrogate PUVA-associated short-term skin damage. Six to 8 wk old C3H/HeNCr mice were painted on their shaved backs with 8-methoxypsoralen (100 ug per 100 ul ethanol) 30 min before exposure to 1 minimal phototoxic dose of UVA (i.e. 50 kJ/m2). The inflammatory response to PUVA was monitored by skin swelling measurements and skin samples were taken from the PUVA-exposed dorsal skin of mice at day 1, 2, 3, 5 and 15 after exposure for microscopic examinations, including H/E, TUNEL, and immunohistochemical p53 stainings. The intraperitoneal administration of both the PAF receptor antagonist PCA-4248 or the selective COX-2 inhibitor SC-236 (0.2 ug/100 ul each; 15 min before UVA treatment) were highly effective in preventing PUVA-induced phototoxic skin swelling response. Both drugs also inhibited PUVA-induced sunburn cell formation, cellular skin infiltration, and epidermal hyperplasia. In addition, both drugs partially inhibited PUVA-induced p53 upregulation and apoptosis (i.e. markers of cellular damage). These results indicate that the PAF pathway is involved in PUVA-associated skin damage and apoptosis. The intervention in the PAF pathway may offer new avenues in preventing the undesired side effects of PUVA.

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Crosstalk of ultraviolet (UV) B and UVA radiation-induced signaling: a complex relationship

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In previous years, the signaling pathways responsible for UVB and UVA radiation-induced gene expression have been characterized in great detail. In particular, UVBR-induced gene expression in human epithelial cells requires the activation of the MAPKs ERK1/2, p38 and JNK, whereas UVAR-induced gene expression is initiated by second-messenger ceramide and subsequently mediated by activation of transcription factor AP-2. Since human skin is mainly exposed to UV radiation from natural sunlight that consists of both UVB and UVA radiation, we have asked in the present study whether these two signaling pathways are independent of each other or whether and how they can interact. We have observed that the strong activation of ERK1/2 in UVB-irradiated primary human keratinocytes can be completely blocked if the cells are preirradiated with UVAR. In contrast, UVBR-induced activation of p38 and JNK was significantly enhanced by preirradiation with UVA. Identical results were obtained if the sequence of irradiations was reversed, i.e. if UVB irradiation was followed by UVA exposure. The observed crosstalk between UVA and UVB-induced signaling was most likely mediated by second-messenger ceramide, since pretreatment of cells with cell-permeable C2-ceramide was capable of mimicking the UVA effect on the UVB response, i.e. attenuation of ERK1/2 activation and stimulation of p38 and JNK activation. These studies demonstrate for the first time a crosstalk between UVB and UVA radiation-induced signaling in human cells. We believe that our observations provide the molecular basis for previous and contradictory reports on agonistic and antagonistic effects of combined UVA and UVB irradiations. Further analysis of this crosstalk will be of critical importance for the judgement of widely used photoprotective strategies such as modern broadband sunscreens.

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UVB-induced oxidative DNA base damage in primary normal human epidermal keratinocytes

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To evaluate the effects of ultraviolet-induced environmental trauma on human skin cells, primary normal human epidermal keratinocytes (NHEK) were exposed to ultraviolet B radiation (UVB; 290–320 nm). We found that relatively low doses of UVB (62.5–500 mJ/cm^2) caused dose-dependent increases in 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a biomarker of oxidative DNA damage. Unirradiated NHEK contained 1.49 (± 0.11 SEM) 8-oxo-dG/10^6 2′-deoxyguanosine (dG) residues in cellular DNA, which increased linearly to as high as 6.24 (± 0.85 SEM) 8-oxo-dG/10^6 dG after irradiation with 500 mJ/cm^2. Further, this oxidative damage was reduced by 60.7% when the cells were pre-treated with 1 mM mannitol. Since hydrogen peroxide (H_2O_2) is known to be generated during oxidative stress, its accumulation in UVB-irradiated NHEK was also assessed and correlated to 8-oxo-dG formation. An UVB-induced increase in H_2O_2 was observed in NHEK and its production was inhibited by the addition of catalase. Based on the ability of a neutral molecule like H_2O_2 to permeate membranes, our data indicate that, after UVB irradiation, H_2O_2 migrates from the cytosol to the nucleus where it participates in a Fenton-like reaction that results in the production of hydroxyl radicals (OH^-) which may then cause 8-oxo-dG formation in cellular DNA. This conclusion is supported by our data showing that OH^- scavengers, such as mannitol, are effective inhibitors of oxidative DNA base damage.

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Prevention of UV-induced immune suppression in humans by T4 liposomes, RNA and a multiagent lotion

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DNA damage is involved in UV-induced immune suppression and carcinogenesis. Sunscreens attenuate the UVR that reaches the skin. Once DNA damage ensues, other agents may help in the repair process. T4 endonuclease liposomes excise UV-induced CPDs and have shown efficacy in reducing neoplasia among skin cancer-prone individuals. RNA fragments have not been utilized for photoprotection although previous reports show that DNA fragments are photoprotective. The benefits of antioxidants have also been reported. We aimed to compare the effects of three formulations containing either T4 liposomes, RNA fragments, or a lotion with multiple photoprotective agents, on the prevention of UV-induced contact hypersensitivity (CHS) suppression in humans. 100 volunteers, FST I-III, were randomized into 5 groups which received no treatment, vehicle, or one of the above preparations. Products were self-applied on gluteal skin once daily for 6 days. Half of the subjects per group received solar simulated radiation (SSR) at 0.75 MED on day 3 of product use. DNCB sensitization followed 3 days post irradiation. CHS response was measured via the increase in skin fold thickness over 5 elicitation sites on the arm, 2 weeks after sensitization. Unprotected SSR irradiation resulted in 65% CHS suppression. Suppression was reduced to 35% by T4 endonuclease, and 23% by RNA fragments. CHS response was enhanced by 29% in the group treated with photoprotective lotion, which contained sunscreen, antioxidants and RNA. These results introduce RNA as a photoprotective agent with efficacy against UV-induced immunosuppression that is greater than T4 endonuclease alone, but not as great as a multiagent formulation containing RNA fragments.

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Endogenous tissue autofluorescence accentuates nonmelanoma skin cancer margins

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Tissue fluorescence in the absence of exogenous photosensitizing agents is termed autofluorescence (AF). Normal human skin and nonmelanoma skin cancers (NMSCs) exhibit different patterns of AF when irradiated under defined light conditions. NMSC are associated with increased levels of extracellular matrix proteinases, which may explain their biologic behavior. When lesions are early or previously biopsied or treated, they may be difficult to discern from surrounding normal skin. Biopsy proven NMSC lesions [basal cell carcinoma (BCC), squamous cell carcinoma (SCC)] were irradiated *in situ* with narrowband near-UV visible light [$\lambda_{excitation}=\lambda_{ex}$=400-550 nm]. Resultant AF images were digitally recorded through highband pass filters [$\lambda_{emission}=\lambda_{em}$>450-500 nm] to remove specular reflection and record AF emissions. NMSC lesions were associated with areas of decreased or absent AF against the relatively bright background AF of normal skin. Bacterial porphyrins incidentally highlighted sebaceous structures. AF images were analyzed digitally to determine approximate putative tumor areas. Calculated areas were correlated with post-excision defect sizes prior to repair. λ_{ex}-λ_{em} matrices suggested optimum delineation of apparent NMSC tumor margins at λ_{ex}=400±10 nm and λ_{em}>500±10 nm. Physiologic rationale and potential applications in management of NMSC are discussed.

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Systemic administration of the phototumorigenic flouroquinolone antibiotic lomefloxacin photosensitises thymine dimer formation in mouse skin

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The fluoroquinolones (FQ) are systemic antibiotics used against a broad spectrum of organisms, including *B.anthracis*. Lomefloxacin (LOM) photosensitises human skin to solar UVA radiation (315-400nm) and we have previously shown that a single systemic treatment with 0.1mg LOM is phototumorigenic in mouse skin exposed to UVA. Many LOM photosensitised tumours contained C|T or CC|TT mutations in *p53* which are indicative of thymine dimer (T^T) formation normally associated with UVB (280-315nm) exposure. Although we have shown that LOM is capable of photosensitizing T^T in human skin cells *in vitro*, it is not known whether this occurs after systemic administration of the drug *in vivo*. Hairless albino (Skh-1) mice (n=4) received a single i.p. injection of 0.1mg LOM and their dorsal skin was exposed to 20 J/cm^2 UVA (Waldmann PUVA source). Control animals were exposed to UVA alone (n=4) or to 250 mJ/cm^2 UVB (Philips TL-12; ~270-340nm). Immediately after irradiation dorsal and ventral skin samples were snap-frozen. T^T were visualized in 10µm frozen sections using the H3 monoclonal antibody and standard immunoperoxidase techniques. Sections were randomized, blinded and scored on a 5-point ordinal scale (0= no stain, 4=heavy staining in all epidermal nuclei). The range of mean T^T levels in the ventral (photoprotected) skin of all groups of animals was 0.55-0.61 whilst the UVB irradiated skin scored 2.96±0.18 (mean ± s.e.). UVA alone produced some T^T (0.72±0.05) but this was significantly increased to 1.74±0.12 by systemic LOM ($p<0.0001$). Our results suggest that systemic LOM can reach epidermal DNA and photosensitise T^T lesions which have mutagenic consequences for *p53* and other loci associated with skin tumour development. It is not known whether this will also occur in humans but with recent large-scale prophylactic use of FQ, it is advisable that sun avoidance measures should be adhered to.

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Topical DNA oligonucleotide treatment down-regulates constitutive and UV-induced COX-2 expression in cultured cells and in mouse and human skin *in vivo*

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Overexpression of COX-2 has been demonstrated in chronically irradiated murine skin and inhibition of COX-2 reduces photocarcinogenesis in mice. p53 is reported to regulate COX-2 expression both positively and negatively, with discrepancies likely related to protein activity under different experimental conditions. We have previously reported that DNA oligonucleotides homologous to the telomere sequence (T-oligos) enhance DNA repair capacity (DRC) and induce multiple cancer prevention responses, effects mediated at least in part through up-regulation and activation of p53. To determine if T-oligos affect COX-2 expression, human newborn fibroblasts were pretreated with T-oligos (pTT and GAGTATGAG) or diluent alone for 24 hours, then UV irradiated and processed for western blot analysis. Within 24 hrs T-oligos markedly down-regulated baseline COX-2 expression, coincided with activation of p53 (shown by p53 serine 15 phosphorylation). After irradiation, COX-2 was comparably decreased in diluent and T-oligo-treated samples at 24 hrs. Interestingly, 96 hours post-UV the level of COX-2 was higher in diluent treated cells than in T-oligo-treated cells, this was inversely correlated with p53 levels. To examine the effect of T-oligos on COX-2 expression in vivo, mice were pretreated topically with T-oligo or diluent alone and 24 hrs later UV irradiated. Human skin explants were provided with T-oligo or diluent alone in culture medium and irradiated 24 later. Compared to controls, T-oligo down-regulated constitutive COX-2 immunohistochemical staining in murine and human skin. Pretreatment with T-oligos down-regulated COX-2 expression in irradiated skin (mouse through 16 hours and human skin through 48 hours). We conclude that T-oligos treatment down-regulates constitutive and UV-induced COX-2 expression by activating and up-regulating p53. This effect may contribute to the T-oligo's ability to reduce ptotocarcinogenesis.

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The local immune suppressive effect of experimental UVB phototherapy correlates with the increase of CGRP in UVB-exposed murine skin

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UVB is an effective treatment for skin disorders like psoriasis, in which the cutaneous neurosensory system (CNSS) is suggested to play a pathogenetic role. UVB also induces immune suppression, which may be part of its therapeutic effect. CGRP mediates a range of immunosuppressive effects including suppression of contact hypersensitivity (CHS). We investigated the effects of UVB phototherapy on the CNSS and on CHS to DNFB in hairless mice, and the ability of a broad-spectrum sunscreen to prevent these effects. Skh-1 mice were repeatedly exposed to subinflammatory UVB thrice weekly for 4 weeks. At 1, 3, or 7d after the last UVB exposure mice were sacrificed and the number of intraepidermal nerve fibers (ENF) immunoreactive (IR) for CGRP, SP, or PGP 9.5 was counted, as well as the skin content of CGRP and SP determined by ELISA. At the same time points, other mice were sensitized to DNFB on exposed back skin, challenged on abdominal skin 5d later, and the immune response (i.e. skin swelling) was determined at 24h after challenge. While the number of ENF positive for the common neuronal marker PGP 9.5 in exposed back skin did not change, UVB significantly increased the number of CGRP-IR and SP-IR ENF. The increase in neuropeptide was maximal at 1d after the last UVB exposure and declined thereafter. At all time points, the number of CGRP-IR ENF was 4-5x that of SP. The skin content of CGRP, but not SP, was also significantly increased at 1d after the last UVB exposure. At all time points, CGRP content was 10x that of SP. In addition, UVB significantly reduced CHS to DNFB, also maximal when mice were sensitized at 1d after the last UVB exposure. Sunscreen pretreatment inhibited the effects of UVB on CNSS and CHS. These data indicate that UVB exposure and the resulting increase of CGRP compared to SP in UVB exposed skin may play an important role in the immune suppressive effect of chronic UVB.

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Spatially selective two photon induction of oxidative damage in fibroblasts

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Most methods for studying and treating living skin do not readily discriminate among the three-dimensionally distinct distribution of cutaneous tissue layers and cells. We have previously demonstrated that near-infrared two photon excitation of psoralen can produce DNA adducts in cells as well as in a spatially preferential manner. The method has now been extended to produce reactive oxygen species (ROS) using exogenous photosensitizers. Human dermal fibroblasts grown as monolayer cultures were treated with dichlorodihydrofluorescein diacetate (H_2DCFDA) which acts as both a photodynamic agent as well as a fluorescent sensor of ROS. Individual cells were irradiated with a focused, high intensity 800 nm pulsed laser beam through a microscope objective and then probed to detect fluorescence. Irradiated cells generated significant amounts of ROS relative to background and unirradiated cells. Subcellular irradiation, in which only a fraction of the cytoplasm or the nucleus was irradiated, produced fluorescence throughout the cell during the several minute time scale of the experiment, indicating that focally generated ROS rapidly diffuse throughout the cell and across the nuclear membrane. Finally, to demonstrate the principle that ROS can be spatially controlled in a macroscopic tissue, fibroblasts embedded in a three-dimensional collagen lattice were treated with H_2DCFDA and the photosensitizer doxycycline. The near-infrared laser was focused and scanned in a plane toward the rear of the lattice, and integrated fluorescence from 10 planes throughout the lattice was subsequently quantified. An approximate doubling of ROS near the focal plane of the lattice was observed as compared with the front out-of-focus plane. These preliminary results suggest that two photon excitation may be useful in studying ROS and photodynamic mechanisms in cells and intact tissue, and may eventually be useful in refining the specificity of photodynamic therapy in skin.

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Neuropeptide induction of nitric oxide in human keratinocytes *in vitro*

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Nitric oxide (NO) is an important signaling molecule in both the CNS and the periphery, where it is involved with neurotransmission, vascular and bronchial tone, inflammation and cutaneous immune function. Recently it has been suggested that NO participates in UV-induced immune suppression by mediating Langerhans cell depletion from the epidermis (Yuen KS et. al., 2002). It is not clear, however, by what mechanism NO might do this or how NO interfaces with other UV-induced signaling mediators known to be involved in UV-induced immune suppression such as UV-induced IL-10, CGRP, and TNF-α. For example, CGRP has been shown to be involved in UV-induced immune suppression and erythema. The goal of these experiments was to investigate the possibility that CGRP, known to be upregulated in response to UV exposure, stimulates NO production by epidermal keratinocytes. Exposure of telomerase immortalized keratinocytes to either UVB or CGRP induced NO, but the time course of NO production was different between the two treatments. CGRP from 0.01nM to 10nM was as potent as 1 to 100ng/ml LPS at inducing NO when measured at time points earlier than 24hr. SP and VIP (0.1 to 100nM) were less potent, as was TNF-α. UVB(10-50mJ/cm^2, CGRP and SP(0.1-10 nM) were capable of inducing NO without accompanying apoptosis. NO production resulting from CGRP treatment occurred before iNOS induction. Exposure of keratinocytes to either UVB or CGRP induced iNOS and eNOS but CGRP upregulation of NOS was much less marked than UVB. We conclude that CGRP can stimulate production of NO by two separate mechanisms: an early phase which is independent of iNOS or eNOS protein synthesis, and a later phase which results from iNOS and eNOS upregulation. In vitro, CGRP treated keratinocytes display a time course of NO induction that contrasts to keratinocytes exposured to UVB. Therefore, CGRP stimulation of NO may contribute to UV-induced erythema and immune suppression following UV stimulation of neuronal CGRP release.

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Fructose 1,6-diphosphate prevents UV-induced photoaging in normal human fibroblast and human skin *in vivo*

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Exposure of human skin to ultraviolet(UV) irradiation, the major environmental factor that affects the structure and function of skin, including sunburn, immune suppression, inflammation, cancer and photoaging. The mechanism responsible for skin damage caused by ultraviolet irradiation is not fully understood, however. Oxidative stress is discussed as one of the major contributors. Fructose-1,6-diphosphate(FDP), a glycolytic metabolite, is reported to have cytoprotective effects against ischemia and postischemic reperfusion injury of brain and heart, presumably by augmenting anaerobic carbohydrate metabolism. Recently, we showed that FDP reduced UVB-induced increase in cellular ROS level although it did not show direct radical scavenging effect in the experiment using 1,1-diphenyl-2-picrylhydrazyl(DPPH) and preserved the cellular antioxidant capacity including catalase activity and GSH content after irradiation. On these premises, we examined whether FDP have protective effects on UV-induced photoaging in normal human fibroblast culture model and human skin *in vivo*. UV irradiation increased MMP-1 expression and decrease of collagen expression in fibroblast and human skin, which was significantly blocked by FDP although it did not have direct radical scavenging and UV absorbing effect. And FDP prevents UV induced decrease of cellular GSH contents. It also prevents UV induced NFκB translocation, which are activated by cellular oxidative signal. Our results indicate that FDP has protecting effects in UV-injured skin aging by decreasing UVB-induced MMP-1 expression and procollagen depression, which are possibly through blocking intracellular oxidative stress. Therefore, FDP may be useful as anti-aging agent for cosmetic purpose.

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The osmolyte strategy of human keratinocytes

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Organic osmolytes, such as betaine, myo-inositol and taurine, are accumulated or released in response to hyperosmotic cell shrinkage or hypoosmotic cell swelling, respectively, to maintain cell volume homeostasis. Osmolyte uptake is important for protection, e.g. against oxidative stress. This so called osmolyte strategy requires the expression of specific osmolyte transporting systems such as the betaine-/g-amino-n-butyric acid transporter BGT-1, the sodium-dependent myoinositiol transporter SMIT and the taurine transporter TAUT. Osmolyte strategies have been characterized in detail in liver, kidney and neural cells and found to be tissue-specific. In contrast, nothing is known about osmolytes in the skin. Here we report that primary normal human keratinocytes (NHK) express mRNA specific for BGT-1, SMIT and TAUT. In comparison to normoosmotic (305 mosmol/l) controls, which only expressed TAUT mRNA, significant induction of BGT-1 and SMIT mRNA expression was observed 6h and 24h after hyperosmotic exposure (405 mosmol/l). Expression of osmolyte transporters was associated with an increased uptake of radiolabeled osmolytes. Conversely, hypoosomotic (205 mosmol/l) stimulation induced an efflux of osmolytes. Identical data were obtained in HaCaT cells. Cellular hydration can change after oxidative stress. Since UVB is a source of oxidative stress and UVB and osmotic stimulation induce similar signaling events we next assessed the influence of UVB on osmolyte transport. Exposure to UVB (0-100 J/cm2) significantly stimulated myo-inositol and taurine uptake in NHK and taurine and betaine uptake as well as mRNA expression for BGT-1 and TAUT in HaCaT cells. Osmolyte efflux was not affected by UVB in either cell type. These studies for the first time demonstrate that NHK possess an osmolyte strategy, which is important for their capacity to maintain cell volume homeostasis and seems to be part of their response to UVB.

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Rinse-off-products containing alpha-tocopherol: an efficient strategy of supplementing the skin barrier with vitamin E and protecting it from photooxidative damage

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α-Tocopherol, the major biologically active vitamin E homologue, is generally regarded as the most important lipid soluble antioxidant in human tissues. Several studies demonstrated the protective effects of topically applied α-tocopherol leave-on formulations against immunosuppression, DNA damage, and carcinogenesis. Only little is known about the penetration characteristics and efficacy of α-tocopherol in rinse-off products. The objective of this study was to investigate whether the single use of an α-tocopherol enriched rinse-off product provides i) effective deposition of α-tocopherol and ii) photoprotection of skin surface lipids. To test this, forearm skin of 13 volunteers was washed either with an α-tocopherol enriched rinse-off product (TP) or with an α-tocopherol free vehicle control (CP; contralateral arm) using a standardized wash protocol. Thereafter, skin surface lipids were extracted with pure ethanol at t=0h or 24h. Additionally, some volunteers were subjected to a single irradiation of their forearms with low-dose UVA(8 J/cm^2) before lipid extraction (n=6). Skin lipid extracts were analyzed by HPLC using electrochemical detection for vitamin E and UV-detection for squalene and squalene monohydroperoxide (SqmOOH). Remarkably, the results of this in vivo study demonstrate that 1)CP treatment lowers, TP treatment strongly increases α-tocopherol levels of skin surface lipids 2)increased vitamin E deposition effects were maintained for a period of at least 24 h, and 3)TP treatment provided significant photoprotection of the skin surface lipid squalene. Thus, the use of α-tocopherol enriched rinse-off products may help to maintain the integrity of the environmentally exposed skin barrier.

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New development in photoprotection: Oral supplementation in healthy volunteers prevents the oxidative, inflammatory and immune UV-responses in blood cells

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Sunlight ultraviolet radiation is involved in several types of skin damages including sun burn, photoaging, photoimmunosuppression and photocarcinogenesis that are partly mediated through oxidative pathways. The purpose of this study was to search new ways for photoprotection and to evaluate an oral photoprotective preparation (PP), composed of antioxidants and essential fatty acids. In order to mimic oxidative stress involved in the cellular aging, we treated the peripheral blood lymphocytes of volunteers by TNFα (50ng/ml)or UVA (15J/cm2) in vitro. Then, we analyzed the activation of the transcription factor NFκB and the generation of DNA damage, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OxodG). Our results clearly show the antioxidant properties of the oral PP supplementation: it significantly decreased the UVA-induced 8-OxodG level (8-OxodG/105dG were 0.23 and 0.46 for the PP and placebo group, respectively) and limited also the TNFα-induced NFκB activation (O.D. NFκB were 0.20 and 0.26 for the PP and the placebo group, respectively). As regards skin immunosuppression, we investigated the PP effect on monocyte-derived Langerhans cells (MoLCs) generated in vitro from purified monocytes of volunteers: we focused on the expression of CD86 co-stimulatory molecule which is of crucial importance in the antigen-presenting function of Langerhans cells. Before 15J/cm2 UVA irradiation, MoLCs were supplied or not with PP and then stimulated by 100ng/ml IFNγ. FACSCalibur flow cytometer analysis demonstrated the restoration by the PP of the UVA-induced inhibition of CD86 expression (100% positive effect for cell cultures; 78% positive effect for oral treatment) illustrating its immunoprotective properties. This systemic photoprotection limits the deleterious effects of UV for endpoints biological responses that differ from sun burn. In combination with UV filters, this oral supplementation could provide better photoprotection for human skin relevant to prevent photocarcinogenesis and photoaging.

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Repetitive heat shock does not lead to accomodation in human keratinocytes and protects from UVB-induced cell death

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It has been demonstrated that hyperthermia protects keratinocytes from UVB-induced cell death in culture and in vivo. This effect is mediated by the anti-apoptotic effect of heat shock proteins that are transiently induced after exposure to heat at sublethal temperatures. The aim of this study was to investigate whether repeated exposure to hyperthermia leads to adaptation of the cells, attenuation of the heat shock response, and abrogation of the protective effect. Cells (A431, normal human epidermal keratinocytes) were exposed to 42 C/4 h at 24 h intervals for 4 consecutive days. The intracellular level of the 72 kD heat shock protein (HSP72) was determined prior to and after each heat shock by ELISA. Immediately after the first and the last heat shock cells were exposed to UVB from a metal halide source (400 and 600 mJ/cm^2) and survival was determined 24 h after exposure by an MTT assay. Our results demonstrate that (i) NHEK and A431 are not significantly different with respect to the response to repeated heat shock; (ii) repetitive heat shock has no influence on cell growth over a period of 4 days; (iii) hsp72 is strongly induced after 4 h at 42 C and is still above baseline at 24 h; (iv) re-exposure leads to a superinduction without attenuation of the absolute increase in hsp72 and of its UVB-protective effect. Induction of hsp by heat shock and other inducers has been proposed as a novel means of photoprotection. However, with daily UVB-exposure of human skin in vivo this approach would be less useful if keratinocytes would become less sensitive to hsp-induction with repeated exposure to the inducing agent. Here we show that adaptation does not occur over a period of 4 days in culture, thus providing a basis for the development of non-toxic hsp-inducers that can be repeatedly applied without loss of effect.

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Initial Vienna experience with extracorporeal photochemotherapy in refractory lung transplantation patients: a pilot study

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Because photopheresis(ECP) has been described as an immunomodulatory therapy that can significantly decrease the risk of cardiac transplant rejection without increasing the incidence of infection,a pilot study was designed to evaluate its efficacy to similarly control refractory chronic lung transplant rejection. Patients who were undergoing ongoing refractory lung transplant rejection or bronchiolitis obliterans syndrome were included. In the four patients (mean age 50 +/- 16.3yrs)included in this study ECP was performed on two consecutive days in intervals of two weeks until response was noted or could not be achieved (8 month limit). Response was evaluated by lung function tests including forced expiration volume in one second after maximal inspiration (FEV1), flow after 50% of forced vital capacity or maximal expiratory flow (MED-50) as well as blood gases and repeat lung biopsies as needed. Within the deliniated treatment period limits (2 to 8 months) improvement of lung function was achieved in the first two patients with significant increases in FEV1 169.2% +/- 38.1 and MEF50: 298.3% +/- 16.2; stabilization was observed in the third patient and diminished but continued deterioration in the fourth patient. This small pilot study confirms previous studies and suggests that further evaluation of ECP in larger patients cohorts is justified.A planned prospective, randomized, multicenter study should help confirm these initial positive observations.

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A comparison of photosensitivity disorders in African-Americans and Caucasians

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This study was designed to evaluate and compare the distribution of photosensitivity disorders in African-Americans and Caucasians in an academic medical center. The charts of 148 consecutive African-American or Caucasian patients with the diagnosis of a photosensitivity disorder or patients who had phototesting performed over a five-year period were reviewed. There were 74 African-Americans and 74 Caucasians with photosensitivity disorders. In African-Americans and Caucasians the distribution of diagnoses was: polymorphous light eruption (57%, 40.5% respectively), systemic phototoxicity to therapeutic agents (21.5%, 11%), chronic actinic dermatitis (15%, 7%), solar urticaria (2.5%, 11%), porphyria (0%, 23%), solar pruritus (0%, 4%), photocontact dermatitis (0%, 3%), juvenile spring eruption (0%, 3%), and nonspecific photosensitivity disorder (4%, 1%). The distribution of photosensitivity disorders in African Americans and Caucasians is different. The most common photosensitivity disorder in both races was polymorphous light eruption. African Americans had a higher proportion of systemic phototoxic reactions to therapeutic agents and chronic actinic dermatitis. Solar urticaria and porphyria was seen more commonly in Caucasians.

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Squalene monohydroperoxides induce apoptosis in HaCaT cells

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Squalene monohydroperoxides (SqmOOH) have recently been identified as the most sensitive *in vivo* marker of human skin surface lipid photooxidation. To date, the effects of SqmOOH on epidermal target cells such as keratinocytes are poorly understood. The present study was undertaken to investigate the effects of SqmOOH on intracellular signal transduction processes involved in cell growth and apoptosis. To investigate this, HaCaT cells were stimulated with varying concentrations of SqmOOH or non-peroxidized squalene. Then, the activation of intracellular signaling pathways regulating cell growth and differentiation as well as apoptosis were assessed by propidium iodine and Hoechst 33343 staining as well as by Western blotting. It was demonstrated that high doses of SqmOOH exhibited cytotoxic effects on HaCaT cells, while low doses promoted cell proliferation. SqmOOH induced apoptosis, as assessed by PARP cleavage, by activating a mitochondrial apoptosis pathway involving Caspase 9 and its effector Caspase 3, in a dose- and time-dependent manner. Moreover, it could be demonstrated that ERK and by p38 MAPK pathways were activated by SqmOOH, while the JNK pathway was unaffected. Importantly, the effects of ERK and p38 MAPK inhibitors suggested that these MAPKs are involved in the induction of SqmOOH-induced apoptosis. In conclusion, SqmOOH induce a well defined mitochondrial apoptosis pathway in keratinocytes. Previously described pathways of UVB or UVA induced apopotosis have focused on direct effects initiated in epidermal cells at the site of the cell membrane (death receptor activation), cytosol (reactive oxygen species) or the nucleus (DNA damage). Our data suggest an additional apoptosis pathway with relevance for human skin, which is initiated by the formation of specific photooxidation products at the site of skin surface lipids and, subsequently, their penetration into adjacent cellular layers of the epidermis.

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Keratinocyte proliferative and apoptotic responses to ultraviolet light (UV) in cyclooxygenase-1 (COX-1) knockout mice

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Skin cancer results when keratinocyte proliferation becomes dysregulated and sustained in the epidermis. Prostaglandin E_2 is a key regulator of epidermal growth produced by COX-1 & -2. Selective COX-2 inhibitors very effectively suppress ultraviolet (UV)-induced tumor development and progression. Paradoxically, overexpression of COX-2 in epidermis can suppress chemically induced tumors. Clearly, these studies show prostaglandin (PG) regulation is a prominent mechanism regulating tumor development. These findings also raise the possibility that COX-1, the constitutive enzyme for PG formation has a role in keratinocyte - derived cancer formation. To understand the role of COX-1 in keratinocyte tumor growth, we studied transgenic mice lacking the COX-1 gene. An experiment was done in which COX-1 -/- transgenic hairless SKH mice were chronically irradiated using UVA-340 sun lamps for 15 weeks, 3 times a week for a total dose of 3.81 x 10^3 mJ UVB. Tumor incidence was monitored. An acute experiment was also done wherein transgenic mice were irradiated once with 180 mJ UVB (equal to an hour of summer sun at noon in Rochester, NY). Keratinocyte proliferation and apoptosis in control and irradiated mice were evaluated in both experiments. The results showed that COX-1 -/+ and -/- animals were more susceptible to UV-induced squamous cell cancer, with onset of tumors occurring 6 weeks earlier than in control mice. Apoptosis, measured by TUNEL staining, was decreased 10 fold in these animals while proliferation was less strikingly affected. We conclude that COX-1 -/- & -/+ animals are more prone to develop UV-induced skin cancer due to misregulation of apoptotic and proliferative mechanisms needed in UV repair. The possibility that similar mechanisms may play a role in humans suggests signalling pathways modulated by PGE_2 may be important to skin cancer prevention.

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***All-trans*-retinoic-acid increases UVB-induced apoptosis of keratinocytes in *in vitro* reconstructed human epidermis**

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We have shown in the past, that all-trans-retinoic-acid (ATRA)-treatment of primary keratinocytes (KC) in monolayer-culture leads to an upregulation of several pro-apoptotic caspases, without inducing apoptosis. However, in contrast to untreated KC, KC after ATRA-treatment were highly susceptible to UV-induced apoptosis. Because retinoids may act differently in vitro and in vivo, we studied their effect on differentiating KC-cultures mimicking the in vivo situation. ATRA-treatment of these skin equivalents (SE) by itself induces apoptosis in up to 30% of the cells. UVB-irradiation (30 mJ/cm^2) of ATRA-treated SE further increased KC-apoptosis up to 100%. By contrast, in DMSO-treated SE only about 10% apoptotic KC, restricted to the basal layer, were observed after irradiation. Because p53 plays a central role in UVB-induced apoptosis, we investigated whether ATRA also influences p53-expression of KC. In DMSO-treated SE, p53 was found almost exclusively in basal KC, while after ATRA-treatment p53 could be also detected in suprabasal layers, both before and after UVB-irradiation. This expression-pattern correlated to the distribution of apoptotic KC observed after UVB-treatment. Furthermore, both the p53-inhibitor alpha-pifithrin, and the pan-caspase-inhibitor zVAD strongly reduced UVB-induced apoptosis, indicating that regulation of p53-expression participates in the ATRA-mediated sensitization to UVB. Taken together, our data suggest that in SE suprabasal KC lose their potential to undergo UVB-induced apoptosis. Furthermore, we show that ATRA-treated KC of all epidermal layers are sensitized to UVB-mediated apoptosis, probably via upregulation of p53-expression.

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***In vivo* evidence for increased oxidative stress in localized scleroderma lesions**

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The pathopysiology of localized scleroderma is believed to involve damage to the vasculature, immune activation and connective tissue dysregulation. Oxidative stress is discussed as one of the major contributors, but convincing in vivo evidence is still lacking. We hypothesized that the natural redox balance is disturbed in scleroderma lesions and results in accumulation of lipid peroxidation products (LPO) and oxidative DNA damage. LPO products, such as malondialdyde (MDA) or 4-hydroxynonenal (4-HNE) tie spontaneously to DNA, RNA or proteins and thus lead to oxidative tissue damage. 8-hydroxyguanosin (8-OHG) is a commonly used parameter of oxidative DNA damage. Biopsies of patients with localized scleroderma (n=16) were compared to healthy skin of age-matched patients (n=16). Expression of the antioxidant enzymes copper-zinc superoxide dismutase (CuZnSOD), manganese SOD (MnSOD) and catalase (CAT) was analyzed by immunohistochemical techniques. To detect LPO products and oxidative DNA damage, protein-bound MDA, 4-HNE, and 8-OHG were investigated. In all cases, stained sections were analyzed using densitometric image analysis. In human scleroderma biopsies, a significant overexpression of the antioxidant enzyme catalase was found within the epidermis and dermis when compared to age- and site-matched control tissue. Notably, the lipid peroxidation markers MDA and 4-HNE were significantly increased in scleroderma tissue. LPO products were found not only in epidermal keratinocytes cells, but also occurred in dermal collagen tissue. Furthermore, 8-OHG was significantly overexpressed within the epidermis of scleroderma lesions.The activation of the enzymatic antioxidant catalase as well as the accumulation of lipid peroxidation products and oxidative DNA damage found in localized scleroderma indicate severe oxidative damage. Thus, oxidative stress is very likely to play a pathophysiological role in the progression of localized scleroderma.

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Collagen degradation in UV-exposed human skin: MMP-1 is the major collagenolytic enzyme and high molecular weight fragments are the major degradation products

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Punch biopsies of human skin were obtained one day after irradiation with two minimal-erythema doses from a UVB/UVA2/UVA1 light source and incubated in organ culture for 72 hours. Organ culture fluids obtained at 24, 48 and 72 hours were analyzed for collagenolytic activity and for reactivity with antibodies to matrix metalloproteinase-1 (MMP-1; interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3). High levels of collagenolytic activity were seen in organ culture fluid from UV-exposed skin. MMP-1 was induced in parallel, increasing from less than 100 ng/ml in organ culture fluid from control skin to approximately 1.8 µg/ml in culture fluid from UV-treated skin. While virtually 100% of the detectable MMP-1 in control culture fluid was represented by the latent form of the enzyme, 50% of the enzyme was present as the active form in organ culture fluid of UV-exposed skin. In contrast, there was little MMP-8 or MMP-13 in control organ culture fluid and only a slight increase following UV exposure. Neutralization studies with a blocking antibody to MMP-1 removed 94+±2% of the collagenolytic activity in the organ culture fluid from UV-treated skin. Additional studies demonstrated the presence of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and TIMP-1 (tissue inhibitor of matrix metalloproteinase-1) in control culture fluids but not TIMP-2. Levels of MMP-9 and TIMP-1 were increased following UV-exposure, and MMP-9 was activated. When intact type I collagen was exposed to organ culture fluid from UV-treated skin, the major degradation products were high molecular weight fragments. These findings strongly implicate MMP-1 as the major collagenolytic enzyme responsible for collagen damage in UV-treated skin. The failure of MMP-9 to further degrade collagen fragments may reflect the high affinity of TIMP-1 for this enzyme.

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Induction of p53 and apoptosis in peripheral blood mononuclear cells by different wavebands of ultraviolet A radiation

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UVA1 radiation (340-400 nm) is increasingly employed to treat T-cell mediated skin diseases and induction of T-cell apoptosis was shown to be a key mediator of the therapeutic effect. In the present study we addressed the question whether this effect is specific for UVA1 radiation or can also be induced by biologically equivalent doses of UVA (315-400 nm) or UVA2 (315-340 nm) radiation. A high pressure UV metal halide lamp with different filter combinations was used to generate UVA, UVA1 or UVA2 radiation. Irradiance and relative spectral intensity were determined with a double monochromator. All experiments were done on freshly isolated PBMC of healthy volunteers. Dose response curves for cytotoxicity as determined by MTT assay were constructed for each of the three UVA spectra. The cells were then irradiated with equitoxic doses (LD10, LD25 and LD50) of UVA, UVA1 and UVA2. Sham irradiated cells served as a control. Quantitative analysis of apoptotic and dead cells was performed by flow cytometry and double staining of cells with annexin V and propidium iodide. Expression of p53 was determined by Western blot analysis. Time course experiments revealed significant apoptosis occuring as early as 2 hours after irradiation. Maximum numbers of apoptotic cells were observed between 6 to 12 hours after irradiation with UVA1 inducing much higher yields of apoptotic cells than UVA or UVA2 at all applied doses. P53 expression was increased at a similar level by all wavebands. Our data indicate that different bands of UVA radiation are all capable of inducing apoptosis in PBMC. However, given in biologically equivalent doses UVA1 appears to be substantially more effective in triggering apoptotic cell death than UVA or UVA2.

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Protection from UVB-induced apoptosis—a novel activity of the neuropeptide alpha-melanocyte stimulating hormone

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UVB-induced apoptosis is a tightly regulated and complex biological response which eliminates DNA-damaged cells. Thus, disruption of this process may lead to cellular transformation. We demonstrate that the neuropeptide alpha-melanocyte-stimulating hormone (alpha-MSH) suppresses UVB-induced apoptosis in cultured normal human melanocytes as shown by reduced amounts of oligo- and mononucleosomes and reduced annexin V staining. This effect was not related to enhanced melanogenesis as keratinocytes but not melanoma cells were likewise protected by alpha-MSH from UVB-induced apoptosis. Colony assays revealed that alpha-MSH or its superpotent analogue NDP-MSH did not delay UVB-induced cell death but indeed promoted long-term survival of melanocytes. To elucidate the mechanism of the anti-apoptotic activity of alpha-MSH, we performed DNA content and cell cycle analysis. Alpha-MSH treatment of UVB-irradiated melanocytes did not alter the percentage of cells in any phase of the cell cycle as compared to cells treated with UVB alone ruling out that the suppression of UVB-induced apoptosis by alpha-MSH is due to G1 or G2 phase delay. Alpha-MSH also did not change the protein expression of Fas (APO-1/CD95), Bcl-2 and Bcl-X while Fas ligand was undetectable in normal human melanocytes. In contrast, alpha-MSH treatment of UVB-irradiated melanocytes resulted in a marked reduction in the formation of pyrimidine dimers as shown by South Western dot blot. Our results highlight a novel biological activity of alpha-MSH in pigment cell biology. Moreover, these findings may explain the biological consequences of the recently reported loss of function mutations of the melanocortin-1 receptor in patients with red hair and light skin and melanoma, respectively.

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Cockayne syndrome A and B proteins are present in mitochondria and involved in the repair of oxidative mtDNA damage

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Cockayne Syndrome (CS), showing neurodegeneration, photosensitivity and premature aging, is caused by mutations in the CSA and CSB gene. Recent evidence suggests that the CSB protein participates in the repair of oxidative DNA damage in the nucleus. Mitochondria (mt) are the cells locus with the highest turnover of reactive oxygen species (ROS). ROS-induced mutations of mtDNA play a role in (i) the normal aging process and (ii) neurodegenerative mt-syndromes similar to CS. We have assessed if the CSA and CSB protein are localized in the mt and whether they may play a role in protecting the mt genome against oxidative stress. Western blot analysis of protein extracts from mitochondria, purified from normal human fibroblasts and free of nuclear contaminants, revealed that the CSA and CSB proteins were detectable in the mt. In marked contrast, CSA and CSB were undetectable in mt-extracts of CSA and CSB patients. We previously demonstrated that mt DNA mutations can be induced in human fibroblasts by repetitive exposure to UVA radiation as a source for oxidative stress. To assess the potential relevance of CSA and CSB proteins for protection of the mt genome we compared the generation of mt DNA mutations in UVA-irradiated fibroblasts from normal individuals and CS patients. UVA irradiation induced mtDNA mutations after 36 repetitive exposures in normal controls, whereas only 12 irradiations were required in CSA and CSB cells. Moreover, stable transfection of the CSB protein into CSB-deficient cells not only restored presence of the CSB protein in their mt, but also normalized their susceptibility towards UVAR-induced mtDNA-mutagenesis. These results indicate a role of the CSA and CSB protein in the repair of oxidative damage in mtDNA and provide the first evidence for their role in the process of aging.

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Inducible DNA repair responses in keratinocytes following differentiation and UV radiation

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Repair of damaged DNA is important for replicative fidelity and successful gene expression. Although differentiating keratinocytes no longer replicate, we have hypothesized that these cells must still maintain global DNA repair mechanisms to ensure all necessary genes are expressed to complete differentiation. Using an immunoblot assay, we have demonstrated that, well after Ca^{2+}-induced terminal differentiation has begun, cultured human keratinocytes maintain global genomic repair of UV-induced cyclobutane pyrimidine dimers and 6-4 photoproducts that is as efficient as in undifferentiated cells. To elucidate mechanisms responsible for the persistence of repair in the differentiating state, we used Western immunoblotting to measure levels of p53, a key mediator of global genomic nucleotide excision repair. In contrast to undifferentiated cells, basal levels of p53 were significantly reduced and not detectably induced in the first 24 hours following 10 J/m^2 UVC in differentiated keratinocytes. Using quantitative real-time RT-PCR, we next quantified mRNA encoding nucleotide excision repair proteins involved in global genomic repair. In undifferentiated keratinocytes, XPA, XPC and DDB2 mRNA were induced by 1.4-, 1.4, and 2-fold, respectively in the first 24 hours following UV, and gradually decreased thereafter. In differentiated keratinocytes, XPA, XPC and DDB2 mRNA were also comparably induced, although the basal levels were reduced relative to total RNA. These results indicate that both undifferentiated and differentiated keratinocytes induce mRNA encoding key DNA repair proteins involved in damage recognition following UV radiation. Unexpectedly, efficient global genomic nucleotide excision repair and induction of repair proteins in differentiated keratinocytes occur in spite of a severe reduction of p53, suggesting other pathways may exist to induce and maintain nucleotide excision repair in the differentiated state.

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Induction of photoaging-associated mitochondrial DNA mutations by repetitive UVB irradiation in normal human keratinocytes

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Mutations of mitochondrial (mt) DNA play a role in the process of photoaging. The most frequent mutation of the mtDNA, the so called common deletion (CD), which is considered to be a marker for mtDNA mutations, has been reported to be induced in-vivo in the dermal compartment of normal human skin as well as in-vitro in normal human fibroblasts by repetitive exposure to ultraviolet (UV) A radiation. Since it is known that pathways relevant for the process of photoaging can also be induced by UVB radiation in normal human keratinocytes, we asked the question whether low dose repetitive UVB exposure of these cells also leads to the generation of the CD. To determine sublethal conditions for repetitive in-vitro UVB radiation exposure, foreskin-derived normal human keratinocytes were irradiated with doses of UVB radiation ranging from 0, 3, 6, 12, to 24 mJ/cm2 three times daily at four consecutive days per week. Cells exposed to 12 and 24 mJ/cm2 UVB showed a significant decrease in cell number and viability whereas doses of 6 mJ/cm2 and below did not affect the viability of irradiated cells. In the following experiments cells were therefore repetitively exposed to 6mJ/cm2 of UVB radiation. The presence of the CD was determined by semiquantitative nested PCR and subsequent Phosphorimager analysis after each week of repetitive UVB exposure. Repetitive irradiation of normal human keratinocytes three times daily, four times per week with 6mJ/cm2 of UVB lead to the induction of the CD after the first week of UV exposure with signal intensities increasing up to the 3 weeks timepoint. These stduies for the first time demonstrate that the CD can be induced in normal human keratinocytes by repetitive UVB irradiation. This observation indicates that mt DNA mutations are not only involved in the process of photoaging induced by UVA but also by UVB radiation.

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Ultraviolet irradiation activates NF-κB pathway via association of IκB kinase-γ/NEMO with IκB kinase-β and IκB-α in human keratinocytes

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Gene expression of primary cytokines IL-1β and TNF-α is transcriptionally regulated by the transcription factor NF-κB. NF-κB activity is sequestered in the cytosol by its association with inhibitory protein IκB. NF-κB activation by soluble inflammatory mediators involves phosphorylation of IκBα (the major IκB protein in human keratinocytes) by IκB kinase-β (IKK-β), which targets IκBα for degradation, and allows NF-κB to localize in the nucleus. Phosphorylation of IκB by IKK requires the scaffolding protein IKK-γ/NEMO. We have investigated the role of NF-κB in UV-induced IL-1β and TNF-α expression and the mechanism by which UV activates NF-κB pathway in human keratinocytes. UV irradiation (30mJ/cm^2, UVB/A2 source) induced IL-1β and TNF-α mRNA in human skin *in vivo* (8-10 fold, n=6) and keratinocytes *in vitro* (3-5 fold, n=4) within four hours. This induction, in keratinocytes *in vitro*, was completely inhibited by adenovirus-mediated expression of dominant IKK-β, or IκBα, indicating that the NF-κB pathway is required for UV induction of IL-1β and TNF-α. Paradoxically, unlike soluble inflammatory mediators (positive control), UV irradiation did not cause detectable IKK-α or IKK-β activation (measured by IP kinase assay), IκBα phosphorylation or degradation (measured by Western blot), or NF-κB nuclear translocation (measured by immunohistology), or DNA binding (measured by EMSA). However, Western analysis of scaffolding protein IKK-γ/NEMO immunoprecipitates revealed that UV increased association of IKK-β and IκBα (2-fold, n=4, measured by Western blot). The amount of IκBα complexed with IKK-β and IKK-γ/NEMO was a minor fraction of total IκBα. These data indicate that NF-κB pathway is required for UV induction of IL-1β and TNF-α. UV activation of NF-κB pathway, in contrast to activation by soluble inflammatory mediators, is difficult to detect because it is mediated through physical association of a small fraction of IκBα and IKK-β with IKK-γ/NEMO.

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Topical DNA oligonucleotide treatment enhances UV-induced DNA repair capacity in human skin *in vivo*

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UV irradiation causes DNA mutations in skin that may lead to skin cancer. We have shown that treatment of human cells with DNA oligonucleotides homologous to the telomere sequence (T-oligos) increases DNA repair capacity (DRC) through up-regulation of nucleotide excision repair (NER) proteins. To investigate *in vivo* the effect of topical T-oligos (pTT and pGAGTATGAG) on DRC, T-oligos were added to culture medium of human skin explants (obtained from sun protected areas) from donors of different ages (24-77 y.o.). After 24 hrs, explants were UVB irradiated and processed for histologic analysis. Routine histology revealed no changes in unirradiated, T-oligo- or diluent-treated skin for up to 160 hrs. In UV irradiated skin by 16 to 48 hrs TUNEL (+) cells indicating apoptosis were comparable in diluent-treated and T-oligo-treated UV irradiated skin. There was no difference in the number of TUNEL (+) cells in diluent vs T-oligo treated skin. Maximum and comparable cyclobutane pyrimidine dimers (CPDs) were detected by antibody staining immediately after UV irradiation (time 0) in diluent-treated vs T-oligos-treated skin, suggesting no sun screen effect of topical T-oligos. Within 24 hrs 51±9% of cells were CPDs (+) (an average of 3 x40 visual fields) in diluent-treated skin vs 25±5% and 29±2% in pTT and pGAGTATGAG-treated skin, respectively ($p<0.001$). The decrease in CPDs (+) cells persisted for the T-oligos-treated skin and it was also evident by 48 hrs post-UV 7±7 and 4±1 vs 12±2% in diluent-treated ($p<0.05$) and at 72 hrs 0 and 0 vs 7±2% ($p<0.001$). We conclude that topical T-oligo treatment of intact human skin enhances DRC, leading to accelerated CPD removal following UV irradiation. Our studies suggest that topical oligonucleotide therapy of human skin may ultimately prevent UV-induced cutaneous malignancies.

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CHOP/gadd153 expression, caspase-3 activation, and sunburn cell formation (apoptosis) are up-regulated concurrently following UVB irradiation of murine skin

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CHOP/gadd153 (or simply CHOP) is a transcription factor, known to be induced by stresses and toxins that damage membranes and cause protein unfolding in the ER. CHOP expression is induced by the same spectrum of damaging agents (toxins, chemotherapeutics, and UV light) that also kills cells by apoptosis, and forcible overexpression of CHOP in cultured keratinocytes and other cell types has been shown to induce apoptosis directly (Maytin et al., Exp. Cell Res. 267:193-204, 2001). We wondered whether CHOP after UVB exposure may participate in UVB-induced apoptosis in vivo. We exposed SKH-1 hairless mice to single doses of UVB (80 mJ/cm2), and the time course of CHOP expression was followed in frozen sections using anti-CHOP antisera and a Cy3-conjugated secondary antibody. CHOP increased slightly at 30 min, more at 4 h, and very strongly at 24 h as compared to the sham-irradiated flank of the same animal. This time-course corresponded closely to that for increased epidermal SBC. These changes were confirmed in paraffin-fixed skin sections, after epitope unmasking with Retrieval-All solution (Signet Labs; pH 9.5, 30 min, 95 C), which also confirmed the nuclear location of CHOP. Previous studies in keratinocytes in vitro had shown UVB-induction of CHOP mRNA, but our results now demonstrate CHOP induction at the protein level in the intact epidermis. If CHOP has a role in epidermal apoptosis, it might be expected to affect one or more caspases in epidermal cells. Using an antibody that detects cleaved caspase-3 (but not the proenzyme) in paraffin sections, we observed an increase in activated caspase-3 at doses and times similar to those for CHOP and SBC. Caspase-3 represents a downstream target for several caspase pathways, which suggests that UVB-induced apoptosis may be mediated, at least in part, through a mechanism in which CHOP acts via one or more caspase pathways.

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UVA and UVB decrease the expression of CD44 and hyaluronate in mouse epidermis which is counteracted by topical retinoids

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CD44 is a transmembrane glycoprotein and the principal cell surface receptor of hyaluronate (HA). In a recent study we have shown that two major functions of CD44 in the mouse skin are the regulation of keratinocyte proliferation in response to extracellular stimuli and the maintenance of local HA homeostasis. Recently we have shown that the topical application of retinaldehyde (RAL) resulted in an epidermal hyperplasia and a marked increase of CD44 expression in the epidermis of C57BL/6 and SKH1 hairless mice. Our previous studies have also shown that topical RAL prevented the generation of oxidative stress induced by UVA and UVB irradiation in SKH1 mice. In this study we examined, by anti-CD44 antibody and hyaluronate binding protein (HABP) stainings, the expression of CD44 and HA in mouse epidermis pretreated topically by 0.05% RAL, 0.05% retinoic acid (RA) and 0.05% retinol (ROL) for 3 days and irradiated with UVB, and pretreated by RAL and irradiated with UVA. We also explored the RNA expression of CD44 by Northern blot. Immunostaining of vehicle-treated back skin of SKH1 mice revealed the standard membrane localization of CD44 and HA in basal and suprabasal keratinocytes. CD44 expression was significantly reduced in the membrane and became cytoplasmic 2h after UVA (10 j/cm2) or UVB (1 j/cm2) irradiation and reconstituted within 8 or 24 h for UVA and UVB, respectively. HA staining and CD44 RNA expression showed a similar pattern of decrease and reconstitution. Topical application of RAL significantly increased the epidermal thickness and the membrane staining of CD44 and HA in follicular and interfollicular keratinocytes and prevented the decrease of CD44 and HA in the epidermis induced by UVA and UVB irradiation. RA and ROL, as RAL, increased the expression of epidermal CD44 at the protein level and of epidermal HA. The role of retinoid-induced increase of epidermal CD44 and HA in the protection of oxidative stress induced by UVA and UVB irradiation is yet to be elucidated.

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Ultraviolet irradiation induction of Smad7 via activation of transcription factor AP-1 contributes to loss of procollagen gene expression in human skin fibroblasts

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Smad7 functions as an endogenous negative regulator of TGF-β/Smad signaling, which promotes collagen production. Reduced expression of Smad7 is associated with increased TGF-β/Smad activity in fibrotic skin diseases such as scleroderma. UV irradiation impairs TGF-β/Smad signaling and reduces expression of procollagen in human skin *in vivo* and cultured human skin fibroblasts. We investigated the role of Smad7 in UV impairment of TGF-β/Smad signaling and reduced procollagen expression. UV irradiation (solar simulator, 2MED) induced Smad7 mRNA and protein in human skin *in vivo* and cultured human skin fibroblasts. Maximal induction of Smad7 mRNA (2.5-fold, n=3) and protein (3-fold, n=3) was observed within 5 hours post UV. Smad7 protein remained elevated for at least 24 hours after UV. UV irradiation of cultured human skin fibroblasts stimulated Smad7 gene transcription, as determined by Smad7 promoter reporter assay. Smad7 promoter contains both AP-1 and Smad3 response elements. UV irradiation induced protein binding to the AP-1 element, but not the Smad3 element, in electrophoretic mobility shift assays. Increased AP-1 binding resulted from UV induction of AP-1 components c-Jun and c-Fos. Deletion of AP-1 binding site of the Smad7 promoter abolished UV-activation of Smad7 transcription. Over-expression of dominant negative c-Jun also substantially reduced UV induction of Smad7 transcription. Finally, overexpression of Smad7 in human skin fibroblasts resulted in near total inhibition of TGF-β-induced type I collagen α2 promoter activity. Taken together, these data indicate that UV irradiation induces Smad7 via activation of transcription factor AP-1 and that Smad7 contributes to reduction of procollagen gene expression observed in UV-irradiated human skin.

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Photomodulation of human skin fibroblast activity *in vitro* and *in vivo*

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This study evaluated the effects of light emitting diode (LED) photomodulation of human fibroblast cells in tissue culture and also correlated these findings with skin biopsies from subjects who received the same LED photomodulation in a multi center clinical trial. Control and treated fibroblast cells were evaluated using ELISA, microarrays and RT-PCR. Skin biopsies from the faces of photoaged female subjects were obtained at intervals of 1, 4, 8, 12 and 16 weeks past completion of LED photomodulation and established using immunofluorescence. Blinded expert grader of clinical photography, digital skin profilometry and high-resolution ultrasound were also performed to objectively correlate the changes in the clinical appearance of the skin with the structural changes observed with the histopathology. One week after final treatment, the global improvement in appearance of skin in periorbital region was 62% and upper lip improvement was 36%. Other observations in periorbital area included reduction of 27% in skin roughness, 30% in elastosis, 14% in pore size, and 25% in redness. Immunofluorescence showed changes in a variety of structural proteins such as collagen I and procollagen I. Increases of these proteins were observed in both skin biopsies and fibroblast tissue culture while MMP-1 was decreased. Increases in collagen I and decreases in MMP-1 were observed diffusely throughout the dermis. This is in contrast to the upper dermal grennz zone typically associated with ablative thermal photorejuvenation. Traditional non-ablative and intense pulsed light skin photorejuvenation procedures produce a thermal injury to the dermis, however LED photomodulation operates via a non-thermal process. The implications of data from these in vitro and in vivo studies and photomodulation cell signaling theory and clinical skin rejuvenation will be summarized.

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Skin protection by inhibitors of photosensitization reactions

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Reactive oxygen species (ROS) are widely accepted as crucial mediators of skin phototoxicity but the targets of solar radiation leading to the generation of ROS are still poorly understood. We have identified two potentially important targets that interact with UVA to generate ROS within human skin, namely protein-bound advanced glycation endproducts (AGE) and enzymatically formed extracellular matrix (ECM) chromophores such as the collagen crosslink pyridinoline. Generation of the ROS superoxide and hydrogen peroxide was driven by solar simulated irradiation (SSL; 2.30 J/cm^2 UVA, 0.12 J/cm^2 UVB) of skin proteins that included type I collagen and elastin, resulting in pronounced inhibition of proliferation of cultured human HaCaT keratinocytes and CF3 fibroblasts. Glycation also strongly enhanced the sensitization potency of ECM-proteins. Most of the photosensitization was attributed to the UVA-component. We hypothesize a complex synergism between UVA-irradiation and skin chromophores that are present endogenously or result from glycation that results in a vicious cycle of ROS generation underlying skin pathology. The involvement of AGE and other skin chromophores as key mediators of skin photodamage makes them novel targets for therapeutic intervention. In an effort to facilitate the pharmacological development of compounds for topical skin photoprotection, we have developed assays for the identification of promising compounds that can prevent UVA induced generation of different classes of ROS. Two classes of compounds have been identified that confer photoprotection by inhibiting protein glycation or by directly quenching photoexcited states that result from the interaction of chromophores with UVA. The topical delivery of these compounds as novel classes of photoprotective agents could have impact in limiting various skin pathologies including chronological aging, photoaging, diabetic complications, and cancer.

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Contribution of pyrimidine dimers to UVA mutagenesis

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While it is well established that UVA is a mutagen and skin carcinogen, its mechanisms of mutation formation are not well understood. Since UVA, in contrast to UVB, is limited in its ability to generate pyrimidine dimers, UVA-induced oxidative DNA damage has been suggested to mediate UVA-mutagenesis. However, a detailed analysis of UVA- and UVB-induced somatic mutations has not been described in human cells. In order to investigate mutation formation by UVA and UVB, we used the HPRT-mutagenesis assay with primary human fibroblasts. This assay measures the frequency of spontaneous or induced hprt-mutations by selection for 6-thioguanine-resistance. In 3 to 5 independent experiments, we found a mutation frequency of 54 ± 17 hprt-mutants/10^6 cells with 20 J/cm^2 UVA1 and of 20 ± 9 hprt-mutants/10^6 with 20 mJ/cm^2 UVB. To further characterize UVA1- and UVB-induced mutations, we performed nested RT-PCR and sequence analysis of the hprt-gene of UVA1- and UVB-induced hprt-mutants. All mutations were confirmed by sequencing the hprt cDNA in two directions. In a total of 29 sequenced independent mutants, 90 % carried single base substitution mutations, the remaining deletions. 70 % of UVB-induced point mutations were C to T or CC to TT transitions. This is consistent with a predominant role of pyrimidine dimers in UVB-mutagenesis, as these mutations are considered 'signature mutations' for pyrimidine dimers. A hotspot with 12 C to T transitions was observed at bp 659, which is located within a pyrimidine run of CCTTTT, a likely target for pyrimidine dimer formation. Surprisingly, at the same hotspot, UVA1 generated C to T transitions with a similar high frequency (89 %). This points to a mutagenic role of pyrimidine dimers also with UVA1. The second most common mutation was a T to C transversion, possibly arising from thymine oxidation. It was also seen in similar frequency with UVB and UVA1 (20 and 17 %). Our data suggest similar mechanisms of mutation formation, including a prominent role of pyrimidine dimers, with UVA1 and UVB.

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Structural identification and biological significance of a novel UVB-generated platelet-activating factor activity

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Through its ability to stimulate cytokine production and cytotoxicity, ultraviolet B radiation (UVB) has profound effects on human keratinocytes. UVB can also act as a pro-oxidative stressor. Platelet-activating factor (1-alkyl-2-acetyl glycerophosphocholine [GPC]; PAF) is a lipid-derived mediator with many biological effects synthesized by the subsequent actions of phospholipase A2 and acetyltransferase in response to many stimuli. In addition to this enzymatic pathway, recent studies suggest that oxidative stress can directly act on GPC resulting in the formation of GPC with short sn-2 fatty acids, which act as potent PAF-R agonists. We have previously shown that UVB-mediated production of the cytokines TNF and IL-8, as well as UVB-induced cytotoxicity is enhanced by the epidermal PAF-R (J. Biol. Chem. 273:18891; 274:26917). Thus, the objective of the present studies was to structurally characterize UVB-induced PAF activity and define its biological significance. Retroviral-mediated overexpression of the PAF-R in primary cultures of human keratinocytes resulted in enhanced UVB-induced apoptosis both in vitro, as well as in vivo using xenografts of transduced cells grown in SCID mice. To characterize UVB-induced PAF agonistic activity, we subjected lipid extracts from UVB-irradiated epidermal cells to gas chromatography-mass spectrometric analysis. Significant levels of 1-hexadecyl 2-acetyl GPC (PAF), as well as 1-hexadecyl-2-butanoyl and 2-butenoyl GPC were found in epidermal cells following UVB treatment. Finally, UVB treatment of purified 1-hexadecyl-2-arachidonoyl-GPC generated the same molecules directly, indicating a non-enzymatic process. These studies suggest that the PAF system in human skin is a target for UVB, resulting in enhanced cytokine production and apoptosis through the production of novel PAF agonists.

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8-methoxypsoralen photoadducts mapping of p53 gene in the fibroblasts treated with PUVA

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Photochemotherapy with 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) radiation (PUVA) has been used for many years as an effective treatment for psoriasis. However, prolonged use is associated with an increased risk of squamous cell carcinoma (SCC). Analysis of mutations in the p53 gene in DNA isolated from these SCCs revealed a prevalence of non-PUVA type mutations. To resolve the apparent lack of a link between PUVA type DNA damage, the observed mutations and the increased incidence of SCC, 8-MOP photoadducts were mapped at nucleotide resolution using single strand ligation mediated PCR (sslig-PCR). Primary human fibroblasts were treated with 8-methoxypsoralen and either ultraviolet radiation or visible light. Genomic DNA was isolated and exons 5 and 7 of the p53 gene were mapped. Photoadducts were detected at twenty nucleotide sites in exon 5 and at seventeen nucleotide sites in exon 7. In contrast to previous observations that 5'-TpA-3' was the preferential site for photoadduct formation in DNA fragments when dissolved in aqueous solutions, we found that there is a diverse distribution of sites for photoadduct formation at pyrimidines as well as purines.

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Differential effect of NFκB on the response of normal and immortalized human keratinocytes to UVB irradiation

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The transcription factor NFkB has been implicated in the survival of cells following cytotoxic stress. We have examined the role of NFkB in human keratinocytes in response to UVB irradiation. We demonstrate that UVB irradiation induces NFkB activation in a dose-dependent manner. NFkB activation was analyzed by in vitro DNA-binding assays and functionally by in vivo reporter gene assays. UVB activated NFkB in both normal human keratinocytes and immortal HaCaT keratinocytes in a similar manner. The role of NFkB in the response of keratinocytes to UVB irradiation by the introduction of the mutant super repressor IkB gene (IkBsr) into the keratinocytes. The IkBsr prevents the activation of NFkB following cell stress. Suppression of NFkB activity in normal keratinocytes and HaCaT cells resulted in differing effects following UVB-irradiation. As previously described for other cell systems, inactivation of NFkB activity in normal human keratinocytes resulted in an increase in sensitivity to UVB-induced apoptosis. In contrast to these data, inactivation of NFkB in HaCaT cells protected cells from UVB-induced apoptosis. As the induction of NFkB activity by UVB in each cell type was similar, differences in downstream effectors of the NFkB pathway results in pro-apoptotic pathways in immortal keratinocytes and pro-survival pathways in normal keratinocytes.

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Identification of novel genes encoding melanocyte/melanoma-specific proteins using GeneChip

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For understanding of biology of melanocytes and development of diagnostic and therapeutic methods for hereditary and acquired pigment disorders as well as melanoma, we have attempted to identify melanocyte specific molecules systematically using DNAChip. cDNA profiles of various tissues and cell lines including primary cultured melanocytes and highly pigmented melanoma cell line, SKmel23, produced with GeneChip containing 42,000 probes were compared. The 603 genes that expressed in primary cultured melanocytes more than 3 times than other normal tissues were first selected. Among 603 genes, 44 genes predominantly expressed in cDNA libraries made from melanocyte-containing tissues in NCBI database were selected, and actual tissue expression was evaluated by RT-PCR. Two uncharacterized genes (FCRL/FREB and novel gene, MLG: MDR like gene) were preferentially expressed in primary cultured melanocytes, melanoma cell lines, and melanoma tissues. FCRL/FREB has recently been reported to be a member of the Fc receptor family and expressed predominantly in B cells in germinal center. We found that the FCRL/FREB was also expressed in some of the melanoma cell lines and tissues by immunohistochemical analysis with murine polyclonal Ab specific for FCRL/FREB that we have generated. The FCRL/FREB protein made by bacterial and mammalian expression systems were recognized by IgG Ab in sera of melanoma patients and healthy individuals. The other identified novel gene, MLG, appears to encode a 131 aa protein homologous to multi drug resistant gene 1 (MDR1) containing a ATP binding site. The GFP fused protein is localized in nucleus of melanoma cell lines. These molecules preferentially expressed in pigment cells may be useful for further understanding of melanocyte biology and development of diagnostic and therapeutic methods for pigment disorders.

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A double-blind randomized trial of 5% ascorbic acid versus 4% hydroquinone in melasma

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Melasma is an acquired hyperpigmentation of difficult treatment. Sixteen female with idiopathic melasma were included in our trial. After randomization from another clinician they were instructed to use at night, for sixteen weeks, 5% ascorbic acid cream in one side of the face and in the other side, 4% hydroquinone cream. Sunscreen was applied daily throughout the period of observation. They were evaluated every month with colorimetry, digital photography and regular color slides. Subjective evaluation by each patient was taken in account. The best subjective improvement was observed on the hydroquinone side with 93% of good and excellent results compared with 62.5% on the ascorbic side ($p \leq 0.05$), with colorimetric measures there were no statisfical differences. Side effects were present in 68.7% with hydroquinone vs 6.2% with ascorbic acid. Even though hydroquinone showed the best response, ascorbic acid may play a role in the therapy of melasma since it is almost devoid of side effects or it may be part of a combined therapy.

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Human epidermal melanocytes function in reconstructed mouse hair model

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Undifferentiated epidermal cells and dermal cells from newborn mice reconstruct hair follicles effectively when transplanted to the back skin of immunodeficient mice (nude mice: Balb-c nu/nu). Utilizing this system, we investigated whether xenografted human melanocytes are functionally incorporated in murine hair follicles. About three weeks after the grafting of epidermal and dermal cells derived from albino mice (ICR), white hairs were observed on the recipient nude mice. When normal human epidermal melanocytes (NHEM) were added to the above grafting mixture, the reconstructed hairs became gray. Histochemical and immunohistochemical analysis revealed melanin pigments in the reconstructed epidermis and hair follicles, and xenografted human melanocytes were observed around mouse dermal papilla by using human-specific antibodies. Addition of large amounts of NHEM in the grafting mixture darkened reconstructed hairs. NHEM from different donors showed different tyrosinase activity. When these NHEM were grafted, it seemed that the darkness of reconstructed hairs correlated with the tyrosinase activity. However, the melanocytes were not able to change the gray color of reconstructed hairs to black, as in C57/black mouse. Measurement of the melanin content of reconstructed hair suggested that hair color might depend on the melanin content per g of hair. These results indicate that NHEM functioned in the murine reconstructed hair follicles, and their tyrosinase activity and their numbers in the grafting mixture influenced on the degree of hair follicle pigmentation. This reconstructed hair model should be useful for studying interactions between melanocytes, keratinocytes and dermal papilla cells in hair follicles.

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Only magnesium ascorbyl phosphate and copolymer complexed vitamin C affect *in vitro* melanocyte morphology and in consequence the melanin transfer into keratinocytes

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The addition of stable vitamin C derivative magnesium ascorbyl phosphate to the culture medium of reconstructed epidermis modifies lipid synthesis and in consequence improves its barrier function. When under such conditions, melanocytes are integrated into the epidermal reconstruct they exhibit a round morphology with very little melanin transferred into the neighbouring keratinocytes, whereas in the absence of the stable vitamin C, a normal epidermal melanin unit is restored with dark, melanin transferring melanocytes. To study in more detail the effect of vitamin C on melanocyte morphology and melanin transfer, we evaluated the effect of ascorbic acid, magnesium ascorbyl phosphate, a stable derivative, and two vitamin C/copolymer complexes: vitamin C/vinyl pyrrolidone -vinylimidazole copolymer and vitamin C/sodium stryrene-maleic anhydride copolymer, both at a weight ratio of 5/1 at pH6.Treatment of keratinocyte-melanocyte co-cultures with the two vitamin C copolymer complexes resulted in a dose-dependent reduction of melanocyte dendricity. Melanocytes adopted a bipolar morphology and transferred considerable less melanin into the keratinocytes. Similar observations were made after the addition of magnesium ascorbyl phosphate. Treatment of reconstructed epidermis with these compounds induced a measurable reduction of pigmentation. In all cases, removal of the compounds from the culture medium restored a functional epidermal melanin unit. Under our experimental conditions, ascorbic acid had no effect. These results demonstrate for the first time a mode of action for stable vitamin C compounds on melanocytes that explain at least in part its known depigmenting activity.

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Eumelanin and pheomelanin contents of depigmented and repigmented skin of vitiligo patients

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There are two chemically distinct types of melanin: the red yellow phaeomelanin and the brown black eumelanin. Both types of melanin have been detected in human hair, epidermis and cultured melanocytes. We conducted this preliminary study to quantify levels of both eumelanin and phaeomelanin in depigmented as well as repigmented patches of vitiligo on various treatments. We enrolled five patients of vitiligo for this study. We took biopsies from depigmented as well as repigmented lesions after PUVA therapy. The eumelanin and phaeomelanin contents of the skin biopsies were quantified by high performance liquid chromatography (HPLC). The overall mean concentrations in depigmented lesions were 28.56 ng/piece of AHP and 4.33 ng/piece of PTCA (average AHP/PTCA ratio 5.5). In repigmented lesions mean concentration of AHP was 8.27 and that of PTCA was 10.36 (average AHP/PTCA ratio was 0.85). Depigmented lesions showed predominantly phaeomelanin whereas repigmented lesions after PUVA showed predominantly eumelanin. In complex sequence of events leading to death/inactivation of melanocyte, decreased tyrosinase activity or other factors leading to switch to phaeomelanin must be playing a vital role in pathogenesis of vitiligo. We could detect melanin in depigmented lesion of vitiligo of 5 years duration. It seems some residual melanocytes are still active in depigmented lesions.

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Characterization of melanosomes in murine strains with defective organelle biogenesis

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Hermansky-Pudlack Syndrome is a disease that exhibits genetic and clinical heterogeneity and is characterized by pigment dilution and prolonged bleeding. There are at least 17 murine genes which when defective cause HPS, and in humans 4 genes have been identified. The functions of most of the HPS genes are unknown, but are thought to function in melanosome biogenesis/maturation and secretion. As a step towards understanding HPS gene function, we previously reported our analysis of melanosomes in 10 murine strains that are the models for the Hermansky-Pudlack Syndrome (HPS), and demonstrated that the mutations in HPS cause charactieristic blocks in melanosome biogenesis or secretion. Here we analyzed melanosomes in the skin of the 7 remaining known murine HPS strains (buff, misty, ashen, subtle gray, sandy, muted and mocha) using transmission electron microscopy. We find that most of these strains also have increased proportions of immature melanosomes. The exception is ashen, which appears to have normal melanosomes, but, in agreement with previous studies in cultured melanocytes, has an accumulation of melanosomes intracellularly. We also find that the severity of the defects in melanosome biogenesis varies from no defect to smaller organelle size to severe defects in pigment organization and organelle fusion/fission. We present the additional morphological findings here along with a model, incorporating all of the murine HPS strains, postulating which step of melanosome maturation and secretion each defective HPS gene product affects.

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The expressions of melanogenic proteins in human skin after ultraviolet irradiation

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It has been known that mutual responses between melanocytes and keratinocytes play a crucial role in the UV-induced melanogenesis in human skin. Keratinocytes secrete specific factors, such as α-MSH, ACTH, and PGE2, responsible for melanocyte activation. They activate the cAMP pathway in melanocyte and so increase MITF expression. MITF is known to bind the M box and E box motifs and to stimulate tyrosinase family gene promoter. However, it is not known that how long tyrosinase and TRP-1 activities continue after UV irradiation in vivo. In this study, we established an immunohistochemical staining for specimens which were obtained from UV-irradiated skin in five healthy Korean males on 2nd, 5th, 7th, 28th, 56th days after UV irradiation. As a result, with time tyrosinase, TRP-1, and MITF activities increased until 7 days after UV irradiation and fell down to the basal constitutive level 4 and 8 weeks later. We also showed that tyrosinase increased prior to TRP-1, interestingly. This study provide important informations for the clinical evaluation of the effectiveness of whitening agents.

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Effect of co-culture of melanocytes and endothelial cells on melanogenesis

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Melanocytes are found in the basal layer of the epidermis often near microvascular endothelial cells in the dermis. In order to investigate what role endothelial cells might play in melanogenesis, co-culture systems were developed in which human epidermal melanocytes were grown in culture together with human micro-vascular endothelial cells (HMVEC). Interestingly, in the presence of HMVEC, the melanocytes became more differentiated as indicated by greater length and number of dendritic extensions. Analysis of the secreted melanin content, after 9 days in culture, revealed a 2-3 fold increase in the amount of secreted melanosomes in co-cultures compared to melanocyte monocultures. In addition there was a 2 fold increase in the number of melanocytes after 9 days in co-culture. Nitric oxide (NO) is a product of endothelial cells and also stimulates melanogenesis. When co-cultures were incubated in the presence of a NO scavenger (Carboxy PTIO) there was no increase in melanocyte growth or melanogenesis. Together, the data demonstrated that co-culturing of melanocytes with endothelial cell increased both the growth rate of the melanocytes as well as the secretion of melanin per cell and that this process is NO dependent. This is the first demonstration of the effect of endothelial cells on melanogenesis. This suggests a role for endothelial cells in melanogenesis since the UV-A component of sunlight reaches endothelial cells in skin and is capable of inducing NO production by endothelial cells.

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Accumulation of tyrosinase in the endolysosomal compartment is induced by U18666A

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U18666A, progesterone, and several cationic amphiphilic drugs have been shown to alter the trafficking of a number of intracellular membrane proteins including CD63/Lamp3, insulin growth factor 2/mannose 6-phosphate receptor (IGF2/MPR), and the Niemann-Pick C1 gene product (NPC1) as well as ganglioside GM1. We have examined the effects of these compounds on cultured melanocytes at concentrations that have been shown to effectively alter intracellular trafficking. Treatment of melanocytes with U18666A (2.5uM) or progesterone (15 uM) for 96 hours decreased melanin content an average of 67% as compared to control without lowering the total cellular tyrosinase activity. Steroidal alkaloids that preferentially act on the Sonic Hedgehog signaling pathway showed no related specificity in their ability to decrease pigmentation. In melanocytes treated with U18666A, tyrosinase accumulates in a compartment that contains both lysosome-associated membrane protein-1 (Lamp 1) and mannose 6-phosphate receptor (MPR), and stains with filipin, consistent with cholesterol-laden late endosomes/lysosomes. Our results suggest that tyrosinase, like the NPC1 gene product, traverses a U18666A-sensitive trafficking pathway.

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An examination of photoadaptive response using UV transmission spectroscopy

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A study has been carried out in an attempt to characterise the relative contributions of pigmentation and hyperplasia in the photoprotection of skin. UV transmission spectroscopy was used to collect transmission data in the region 250nm – 400nm. Suction blisters were removed from the lower back of volunteers, and inserted into a thin saline filled cavity within a quartz cuvette. This experimental setup was used to minimise the effects of dehydration. Several skin types were examined, and the transmission spectrum of a tanned area was compared with an adjacent control area in each case. Preliminary transmission data showed a wavelength dependent change in the spectra, with decreases in transmission of the tanned skin compared with the control in both low and high wavelength regions. Decreases in the transmission at wavelengths between 250nm and 260nm indicated increases in the levels of DNA, proteins and amino acids, associated with hyperplasia. Decreases in the transmission at wavelengths above 320nm indicated increases in melanin levels, associated with hyperpigmentation. The transmission T of forward directed radiation of wavelength λ through excised epidermis of thickness t can be expressed as: $T(\lambda,t)=\exp[-\{K_a(\lambda)+c.K_m(\lambda)\}.t]+S(\lambda,t)$ equation 1 where $K_a(\lambda)$ is the absorption coefficient of aromatic amino acids at wavelength λ, and $K_m(\lambda)$ is the corresponding coefficient for melanin; c is the melanin concentration, and $S(\lambda,t)$ is a scattering function. The scattering function could be estimated by measuring the transmission through skin containing no melanin, i.e. vitiliginous skin. The absorption by amino acids, $_a(\lambda)$, is effectively zero between 330nm and 400nm, and the function $c.K_m(\lambda)t$ could be calculated in this region for each epidermal sample. The absorption spectrum for melanin is well known, hence the constant (c x t) could be estimated. It was then possible to calculate $K_a(\lambda)t$ by substitution into equation 1. In this way, the respective contributions to photoadaption by pigmentary and non-pigmentary factors could be estimated.

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Photoadaptation is dose dependent, but not site dependent

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We have previously demonstrated the presence of clear person and body site factors for ultraviolet induced erythema (ANOVA model), and that there is a greater variation between body sites within one individual than between individuals ($p<0.01$). In this study we looked at how photoadaptation affects this variability and how much protection against acute erythema is created by facultative photoadaptation. 15 healthy volunteers were exposed to 5 incremental doses of narrow band UVB (Waldmann 108) 0.3J/cm2–0.43J/cm2 daily for 5 days to 3 body sites — upper back, inner forearm and outer calf. 96hrs after the final dose, the photoadapted sites plus adjacent non-adapted sites were challenged with 5 (21/3) incremental doses of UVB, 119–300mJ/cm2 to arm and back, and 238–600mJ/cm2 to outer calf. Erythema induced by the challenge was measured at 24hrs using contact laser doppler (Moor). Baseline erythema measurements were also taken from unchallenged adapted and non–adapted sites. Although challenge erythema varied between body sites, there was no variation in the percentage reduction of erythema in adapted vs non–adapted skin at each site at each UV challenge dose. This difference was inversely related to the challenge dose ($47 \pm 7\%$ at 300mJ/cm2, $57 \pm 1.35\%$ at 238mJ/cm2, 67 ± 5.51 at 189mJ/cm2). There was no significant difference in baseline erythema between unchallenged photoadapted and non–adapted sites. We conclude that the amount of protection against erythema offered by facultative photoadaptation is dose dependent but not site dependent.

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A novel dose-response assay for pigment studies

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To determine the physiological role of genes such as MC1R in pigmentation, quantitative measures of pigmentation are required. We wish to define 1) time course of photoadaptation, 2) pigmentary dose-response to UVB, 3) time course of pigmentary response and 4) how redheads and non-redheads respond to the same dose of UVB. In determining time course of photoadaptation, an experiment (n=9) showed that an area of skin photoadapted daily on 3 consecutive days with UVB produced less flux (contact laser Doppler) compared to unadapted skin after a 100mJ/cm2 UVB challenge. Mean % reduction in flux was noted on days 3-4 (40% SEM=13.1), day 6 (66% SEM=10.0), days 10-13 (65% SEM=10.7), day 17 (70% SEM=6.1), days 19-20 (26% SEM=18.4) and days 24-27 (18% SEM=13.8). To determine pigment response, 24 subjects were given 4 doses of UVB delivered on 1 occasion to the lower back. Readings up to 9 weeks were performed using the L*a*b* system (Minolta chromameter). A novel approach was employed by first iontophoresing irradiated areas with noradrenaline to exclude confounding influence of erythema. Contact laser Doppler flowmetry then ensured satisfactorily low flux prior to L* readings. Results showed that higher doses of UVB produced darker L* readings. At 1 week, 28, 56, 112, 160mJ/cm2 UVB produced a mean increase in L* units of 0.62 (SEM=0.19), 2.55 (SEM=0.45), 6.19 (SEM=0.73) and 7.99 (SEM=0.84) respectively. Time of maximal pigmentation was 1 week when mean increase was 7.99 L* units (to 160mJ/cm2). By week 5, the mean increase had fallen to 2.52 L* units (SEM=0.5),(lighter). Comparing redheads and non-redheads 1 week post-irradiation with 160mJ/cm2, non-redheads darkened by 9.78 L* units (SEM=1.17) while redheads only darkened by 6.04 units (SEM=0.93)($p=0.02$); redheads were ~40% less pigmented than non-redheads. There was no difference between the 2 groups at 28mJ/cm2 ($p=0.16$). In conclusion, photoadaptation was maximal on days 6-17 post-adaptation, with 65%-70% reduction in erythema, demonstrating a dose-response to UVB with maximal pigmentation at 1 week. We have demonstrated a novel dose-response assay for pigment studies.

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The proteinase-activated receptor-2 mediates phagocytosis in a Rho-dependent manner in human keratinocytes

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The process of melanosome uptake by human keratinocytes (HK) is poorly understood. Recent work shows that proteinase activated receptor-2 (PAR-2) activates signals that stimulate melanosome uptake in HK and that PAR-2 expression is regulated by UV irradiation in vivo. The Rho family of GTP-binding proteins is involved in receptor-mediated cytoskeletal remodeling during phagocytosis. Using Nile red beads in a melanosome uptake model, we show that phagocytosis in HK is Rho-dependent because treatment with toxin B (inhibitor of all Rho proteins), C3 exoenzyme (specific Rho inhibitor) and Y27632 (Rho kinase inhibitor) blocked uptake of Nile red beads in HK. We show that PAR-2 mediated phagocytosis is Rho dependent because Rho kinase inhibitor (Y27632) blocked PAR-2 stimulated phagocytosis in HK. Affinity purification of GTP-Rho (activated Rho) from HaCat treated with PAR-2 activating peptides or trypsin (which activates PAR-2) revealed a rapid activation of Rho (>3 fold). We explored PAR-2 signaling in HK and show that activation of PAR-2 elevates cAMP in a dose and time dependent manner and is pertussis toxin (PT) sensitive. PAR-2 mediated Rho activation was independent of PKA because two PKA inhibitors (H-89 & Rp-8-Cl-cAMP) failed to block PAR-2 dependent Rho activation. The Gα12/Gα13 PT- insensitive G-coupled proteins are known to activate Rho in other cell types. We show that HK express Gα12/Gα13 and that PAR-2 mediated Rho activation is resistant to PT. Microinjection of antibodies to Gα13 block Rho-dependent stress fiber formation in HK suggesting that Gα12/Gα13 might link PAR-2 with Rho. These data are the first to show that PAR-2 mediated phagocytosis is Rho dependent and that PAR-2 signals downstream to Rho and cAMP. These data shed light on the mechanisms of PAR-2 mediated melanosome uptake by HK and point to Rho as a central signaling molecule in skin pigmentation.

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Using cDNA microarray analysis to understand pathways related to Waardenburg syndrome and pigmentation

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Waardenburg syndrome (WS) is an autosomal dominant disorder with an incidence of 1 in 40,000 live births. Patients with WS have sensorineural deafness, pigmentation anomalies, cardiac and skeletal abnormalities and neural tube defects. Many patients have loss of function mutations in Pax3. Mutations of Pax3 have been identified in splotch (sp) mouse strains and these mice have characteristics similar to WS patients. Many of the organs and tissues that are affected in WS patients and splotch mice are derived from neural crest cells. Another mouse strain that has defects in tissues derived from the neural crest is the Hoxa3 knockout mouse. Because mice homozygous for Pax3 and Hoxa3 mutations have very similar phenotypes, we investigated whether Pax3 and Hoxa3 lie in the same pathway and regulate some of the same genes. We used semi-quantitative RT-PCR and cDNA microarrays to study specific mRNA levels in pools of embryos derived from Pax3 and Hoxa3 mutant mice. Using RT-PCR, we observed that Hoxa3 was downregulated in Pax3 mutant embryos, while Pax3 was unchanged in Hoxa3 mutant embryos. cDNA microarray analysis revealed that the melanocyte gene, Tyrosinase related protein 2 (Trp-2) is downregulated in both types of mutant embryos. We also observed expression of Hoxa3 in the B16 melanoma line and in melb-a, a melanoblast line. These results suggest that Hoxa3 lies in a pathway downstream of Pax3, and unexpectedly, it may play a role in pigmentation.

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Molecular mechanisms underlying interrupted melanosome transfer in pigmented basal cell epithelioma

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In pigmented basal cell epithelioma (BCE), there is the abnormality of the transfer of melanized melanosomes from proliferative melanocytes to basaloid tumor cells. In this study, to determine molecular mechanisms underlying the disrupted transfer, we used immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) to examine the role of microtubuli-associated motor proteins (MAMP) in melanocytes and of phagocytic receptor, the protease-activated receptor (PAR)-2 on basaloid tumor cells. Electron microscopic analysis revealed that basaloid tumor cells have a significantly lower population of melanosomes than do normal keratinocytes located in the non-lesional epidermis, indicating interrupted melanosome transfer. RT-PCR analysis demonstrated that while MAMP in melanocytes including dynein and kinectin, but not fascin, are markedly downregulated in BCE, phagocytic receptor on keratinocytes, PAR-2 is also slightly, but substantially decreased compared with the non-lesional epidermis. Immunohistochemistry using antibodies to MAMP and PAR-2 demonstrated that melanocytes localized in BCE express no or less immunoreactivity with antibodies to dynein, kinesin and fascin, while basaloid cells exhibit less immunostaining with antibody to PAR-2 than in the non-lesional epidermis. These findings suggest that the decreased expression of these MAMPs in melanocytes as well as PAR-2 in basaloid cells may be associated with the observed interruption of melanosome transfer in BCE.

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Effect of stem cell factor and endothelin-1 on pigmentation in human skin grafted onto SCID mice

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It is known that UVB irradiation causes skin tan, via increasing the activity and number of melanocytes in the epidermis. Previous reports suggest that both stem cell factor (SCF) and endothelin-1 (ET-1) are critically involved in the biological mechanism in this process. In cultured melanocytes, ET-1 and SCF synergistically increase DNA synthesis via induction of MAPK phosphorylation. Nonetheless, little is known pertaining to the mechanism of action of these cytokines in intact skin *in vivo*. The object of this study was to demonstrate the potential effects of SCF and ET-1 on human skin pigmentation using an *in vivo* model, i.e.) human skin grafted onto the severe combined immunodeficient (SCID) mice. We initially examined the effect of the individual cytokines on skin color. Either SCF or ET-1 was injected into the grafted human skin subepidermally at different doses daily for 3 days. To monitor the gross skin color change, L values were measured using image analysis from magnified polarized pictures taken on day 0 before cytokine injection and day 4 before sacrification. SCF injection induced skin darkness significantly in a dose dependent manner. In contrast, ET-1 did not demonstrate a significant effect on gross skin darkness. To determine the synergic or additional effect of SCF and ET-1, both cytokines were injected simultaneously and compared with the effect of individual injections. Neither SCF 0.2μg nor ET-1 0.1μg alone has significantly effected skin darkness. However the combination of both cytokines induced skin darkness significantly. To quantitate melanogenseis, the numbers of dopa-positive melanocytes of treated epidermal sheet were determined. The combined injection of ET-1 and SCF synergistically increased a melanocyte number. In conclusion, these data demonstrated that the SCF and ET-1 synergistically increase human skin pigmentation.

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Fibroblasts play a stimulatory role in keratinocyte proliferation but an inhibitory role in melanocyte growth and pigmentation in a skin equivalent system from skin type 4

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There have been conflicting results regarding the influence of fibroblasts on melanocytes. In this study we investigated the effects of fibroblasts on the number of melanocytes and melanin pigemntation in a skin equivalent model derived from skin type 4. In addition, the effect of fibroblasts on the proliferation of melanocytes in monolayer culture was evaluated using the MTT assay. The skin color in the skin equivalent with fibroblasts (SEF) was much lighter macroscopically than in the skin equivalent without fibroblast (SENF). Histologically, both the SEF and the SENF produced a multilayered epidermis with a compact horny layer. Less melanin, however, was found in the SEF than in the SENF. Immunohistochemical staining with a melanocyte-specific antibody (MEL-5) revealed that the number of melanocytes in the SEF was much smaller than in the SENF. However, in the SEF, the number of Ki-67-positive keratinocytes was increased compared to the number in the SENF. In the monolayer culture, absorbance in the MTT assay by melanocytes with fibroblast conditioned medium was significantly increased compared with that of melanocytes in control culture, suggesting that some types of fibroblast-derived factors may have positive effects on the growth of melanocytes in monolayer systems. Our results show that in a skin equivalent, fibroblasts stimulate proliferation of keratinocytes while reducing the number of melanocytes and suppressing melanin pigmentation, and that in monolayer culture, fibroblasts at least do not suppress melanocyte growth directly.

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Tyrosinase in melanogenic cells might have a protective role against dopamine cytotoxicity

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Tyrosinase is a multifunctional enzyme in the melanin biosynthetic pathway and is primarily expressed in melanocytes. Tyrosinase catalyzes the conversion of tyrosine to 3,4-dihydroxyphnylalanine (L-Dopa) and oxidizes L-Dopa to form Dopa quinone. In addition, it is known that tyrosinase also oxidizes dopamine (DA) to form melanin rapidly via DA quinone. Although the catecholamines DA, norepinephrine, and epinephrine are predominantly synthesized in selected central and peripheral neurons and in the adrenal medulla, human keratinocytes also express tyrosine hydroxylase, which is the first and rate-limiting enzyme in catecholamine synthesis. Therefore catecholamines could be directly produced in the human epidermis by keratinocytes. The pathogenesis of vitiligo has not been completely defined and even the process of melanocytes disappearance from involved skin. Evidence has been accumulated on the role of oxidative stress as the initial event in melanocyte degeneration, and an increase in catecholamine discharge or synthesis has been associated with disease activity. In this study, we explore the physiological role of the tyrosinase enzyme activity toward dopamine. Here we show that 10 μM of DA is significantly cytotoxic to the mouse melanoma cells (B16F10) and that phenylthiourea (PTU), an inhibitor of tyrosinase, dramatically enhances the DA cytotoxicity. We also presents that DA is incorporated into the cells in the time- and temperature-dependent manner. Our results indicate that tyrosinase expressed in melanocytes might have a protective role against dopamine cytotoxicity in the epidermis.

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A new continuous spectrophotometric assay method for DOPA oxidase activity of tyrosinase

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Tyrosinase is a key enzyme in the production of melanin pigment and catalyzes several steps in melanosynthetic pathway. Sensitive assay methods for tyrosinase activity are essential not only for the understanding the process of pigment production but also for the development of effective inhibitors of tyrosinase. To develop an efficient assay method, we applied thymol blue, a pH-dependent indicator, to reaction mixtures. Ultraviolet-visible absorption spectra studies showed that the extent of absorbance change of the thymol blue-applied method was several fold greater than the DOPAchrome formation method. The enzyme kinetic study revealed that DOPA oxidase activity of tyrosinase in thymol blue-applied reaction system was more sensitively measured, even under lower enzyme units compared with the previous report with significant enhancement of Vmax while affinity change on substrate was not observed. To test whether this method could be applicable to the inhibition and the inactivation kinetic study of tyrosinase, the effect of kojic acid, a well-known tyrosinase inhibitor, and sodium chloride respectively, have been studied. Kojic acid showed an inhibitory effect with a Ki value similar to previous reports on tyrosinase activity and sodium chloride inactivated this system in a dose-dependent manner. Conclusively, thymol blue method can assay tyrosinase activity with sensitivity and is applicable to the inhibition and the inactivation study of tyrosinase.

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Non-enzymatic polymerization of melanogenic monomers in keratinocytes by ultraviolet A radiation

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Human skin irradiated with ultraviolet A radiation (UVA) exhibits grayish brown colored pigmentation, which fades rapidly when UVA irradiation is stopped. If the amount of irradiated UVA reaches a certain level, the pigmentation persists more than several hours and the color changes to brown. Some hypotheses were proposed to explain the mechanism of these UVA-induced pigmentations, but it has not yet been clearly elucidated. To know what happened in the skin by UVA irradiation, we excised human skin three hours after UVA irradiation, and found the deposition of melanin in the keratinocytes. Thus we postulated that mechanism of the persistent pigment darkening induced by UVA is photo-polymerization of melanogenic monomers accumulated in the keratinocytes since the reaction occurred in rather a short time. Culture supernatant from normal human melanocyte culture was analyzed using thin-layer chromatography and high performance liquid chromatography. A few colorless spots on a thin-layer chromatography plate turned brown in color responding to UVA. These spots were identified as 5,6-Dihydroxyindole-2-carboxylic acid, 5,6-dihydroxyindole, and their metabolites. We also confirmed that these compounds synthesized chemically were easily turned into melanin by UVA. In vitro study using normal human keratinocytes, co-cultured with melanocytes, showed that UVA induce the darkening of the keratinocytes. These results suggest that melanogenic monomers produced by melanocytes are accumulated in keratinocyte, and polymerized to melanin by UVA irradiation. This reaction might be related to the persietent skin darkening induced by UVA.

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A novel model for post-inflammatory pigmentation

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The post-inflammatory pigmentation (PIP) is frequently observed, but very little is reported on the mechanism of formation because of no experimental model. The pigmentation occurring after acne, as a type of PIP, is a serious concern with regard to appearance. It may be considered to be representative of PIP. Therefore, in order to investigate the mechanism of the PIP, we designed a model by using oleic acid, which is one of the main fatty acids being responsible for acne. First, oleic acid (15%) was applied to the back of brownish guinea pig (1 time/day) for 8 days. Inflammatory condition and color of the skin were observed. Application of oleic acid induced erythema within a day, then desquamation and crust formation occurred. At the day 15 after the application, significantly severe pigmentation in color was observed. At the same time, substantial levels of melanin were detected in the epidermis by Fontana-Masson staining. In addition, levels of prostaglandin E2 (PGE2), an inflammatory mediator, increased at the tested portion of the skin soon after the first application of oleic acid. Then, we conducted in vitro experiments using keratinocytes (KC) and pigment cells (PC) in order to study the mechanism of melanogenesis. We investigated the effects of the oleic acid on PC via KC because it was reported that oleic acid directly inhibited melanogenesis in PC. Melanogenesis in PC was enhanced when KC was cultured with oleic acid and culture medium of KC was added to PC medium. Chemical mediators such as PGE2 were detected in KC medium. We therefore speculated that these mediators stimulated PC and enhanced melanogenesis. These results indicate that PIP is raised by several chemical mediators which are produced in KC after oleic acid stimulation.

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Modulation of heat shock protein 47 expression in aged and photo-aged normal human fibroblasts by *Salix Alba* extract

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HSP 47 is a specific chaperone of pro-collagen. This heat shock protein is responsible for the correct 3-dimentional organization of pro-collagen and its control-quality prior secretion. The aim of the study is to evaluate the level of HSP47 in aged, photoaged and senescent fibroblasts and its modulation by a plant extract (Salix Alba). We measured the level of HSP47 and/or pro-collagen expression in fibroblasts by real time RT-PCR (mRNA transcripts) and by flow cytometry (immuno-chemistry technique for measurement of arbitrary fluorescence intensity). We used immuno-chemistry techniques and confocal microscopy to vizualized the cellular localization of HSP 47 and pro-collagen. These parameters were compared with different age-donors, non senescent and senescent fibroblasts. Fibroblasts were irradiated by a non cytotoxic dose of UVA (6 J/cm2) and HSP 47 level was evaluated. Salix Alba extract was tested for its capacity to modulate HSP 47 expression. We showed colocalization of HSP47 and procollagen by confocal microscopy, indicating that HSP47 could play a role of procollagen molecular chaperone in our cellular model. We showed that HSP47 level is decreased in old-donor cells, senescent and irradiated cells. This decrease can be modulated by a salix alba extract (polyphenols rich) in a dose dependent manner. The evaluation of HSP 47 expression in our experimental conditions can lead to a new approach of aging and photoaging pointing out the implication of this chaperone in these pathophysiologic phenomenons. Modulation of HSP 47 expression by molecules family could be of cosmetic and/or dermatologic interest.

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Mechanisms underlying dysfunction of melanocytes in vitiligo epidermis: role of SCF/c-kit lineage and its downstream MITF-M

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Little is known about mechanisms involved in dysfunction of melanocytes in vitiligo epidermis. We hypothesize that some of cytokine/receptor lineage may be affected, resulting in melanocyte dysfunction and/or loss in the vitiligo epidermis. In this study, we have compared the expression of ET-1, GM-CSF, SCF, c-kit, tyrosinase, S-100α, ETB receptor and MITF-M between the lesional and the non-lesional epidermis using RT-PCR, immunohistochemistry and western blotting. Analysis by RT-PCR and western blotting for ET-1 and SCF indicated rather up-regulated cytokine production by the lesional vitiligo epidermis. Immunohistochemistry with antibodies to melanocytic molecules revealed that in the edge of the lesional epidermis, there are still remaining melanocytes expressing tyrosinase, S-100α and ETB receptor, but not melanocytes with c-kit or MITF-M molecules. Quantitation of the number of immuno-positive cells over 200 basal cells revealed a slight or moderate decrease in the number of S100α, tyrosinase and ETB receptor immuno-positive cells at the edge of the lesional epidermis. In contrast, the number of cells expressing c-kit markedly decreases at the edge of the lesional epidermis compared with the non-lesional epidermis. In the center of the lesional epidermis, there is the complete loss of melanocytes showing c-kit/S-100α/ETB receptor/tyrosinase immuno-positive reactivity. Western blotting using antibodies to c-kit and MITF-M revealed that the vitiligo epidermis is associated with down-regulated expression of c-kit and MITF-M proteins in the edge of the lesional epidermis. These findings suggest that the deterioration of the expression of c-kit on melanocytes and its downstream including MITF-M may be associated with the dysfunction or loss of melanocytes in the vitiligo epidermis.

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MITF regulates PKC-B transcription

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MITF has been implicated as the master gene for pigment cells. MITF is required for maintenance and survival of melanocytes and is a major transcription factor for the rate limiting melanogenic protein tyrosinase. The promoter region of tyrosinase contains M-box and E-box sequences known to bind MITF, allowing gene transcription. MITF expression and function were shown to be increased by a-melanocyte stimulating hormone (α-MSH); and α-MSH or other cAMP elevating agents were shown to increase expression of tyrosinase at least in part by increasing tyrosinase transcription in an MITF-dependent manner. Protein kinase C-B (PKC-B) also plays a critical role in melanogenesis by phosphorylating (activating) tyrosinase protein. To determine whether MITF might also regulate melanogenesis via PKC-B, paired cultures of human melanocytes were transfected with dominant negative MITF (DN-MITF) or control vector and processed for western blotting. PKC-B protein was reduced by 50% within 48 hours in cells transfected with DN-MITF. Tyrosinase activity, measured in parallel, decreased proportionately in DN-MITF transfected vs control cells (5,162 ± 2,467 vs 10,170 ± 604 cpm/μg/hr). Also consistent with a role for MITF parallel to its role in tyrosinase regulation, treatment of melanocytes with the cAMP elevating agent IBMX (100μM) for 48 to 72 hours increased the PKC-b protein level by 2-3 fold. The published sequence of the PKC-B promoter contains an E-box. To further determine if MITF transcriptionally regulates PKC-B, a PKC-B promotor-CAT construct was co-transfected with an MITF expression vector or empty vector into Cos cells that have an undetectable basal level of MITF. Cells were harvested 48 hours later and CAT-assay was performed. Co-transfection of the MITF expression vector compared to the control vector dramatically (60 fold) increased the activity of the PKC-B promoter-CAT construct (8,000 ± 120 vs 120 ± 50 cpm acetylated protein per μg protein in the cell lysate). These results demonstrate that MITF is a transcription factor for PKC-B and support a central role for PKC-B in melanogenesis.

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Bone morphogenetic protein 4: a novel modulator of melanogenesis

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Bone morphogenetic proteins (BMP), members of the transforming growth factor (TGF)-B family, signal in many cells including neural precursors. Two receptors (R1 and R2) coordinately mediate BMP signaling. BMPR1 binds BMP and BMPR2 transduces the signal. BMPR1 has two forms: A and B. Among neural precursors BMPR1A promotes proliferation and BMPR1B promotes differentiation. Using RT-PCR we found that neural crest derived human melanocytes (Mc) express BMPR1A, BMPR1B and BMPR2 and that Mc and keratinocytes (Kc) express BMP4. Furthermore, BMP4 supplementation of Mc decreased melanin/cell by 75±1% ($p<.005$) and increased Mc yield by ∽300% ($p<.03$). In Mc, BMP4 also decreased tyrosinase and PKC-B mRNAs and proteins within hrs of stimulation. BMP is known to act through phosphorylation of cytoplasmic Smad proteins that translocate to the nucleus to interact with proteins like AP-1 to affect gene transcription. Interestingly, the published tyrosinase promoter contains sequences recognized by Smad1 and Smad5 and an AP-1 recognition site flanks the Smad5 site, suggesting that BMP4 down-regulates tyrosinase transcription. To determine BMP4 effect on tyrosinase transcription, AN melanoma cells that, like Mc, express BMP receptors, were transfected with tyrosinase-CAT plasmid construct carrying the full tyrosinase promoter (6.1 Kb). BMP4 decreased CAT activity by 48%±16% (mean±SEM). Also, as known for TGF-B, within 10 min of BMP4 stimulation of Mc, NFKB translocated to nuclei as determined by immunohistochemical analysis and confocal microscopy. Since UV irradiation is the best-recognized environmental stimulator of melanogenesis, Mc and Kc were separately sham or UV irradiated with a solar simulator (20mJ/cm^2 measured at 285±5nm). Within 24 hrs, UV substantially downregulated the mRNAs of the three BMP receptors (Mc) and BMP4 (Mc and Kc) to hardly detectable levels. Within 48 hrs the expression of all mRNAs returned to baseline levels. Our data provide evidence of a novel signaling pathway that influences melanogenesis in human skin.

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Ultraviolet radiation-induced melanogenesis in human skin of different racial/ethnic origin

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We are studying the mechanisms of skin color variation and of pigmentation induction following exposure of human skin of different racial/ethnic origin to ultraviolet radiation (UVR). Previously, we evaluated the usefulness of biomarker methods in response to UVR, including the measurement of UV damage as cyclobutane pyrimidine dimers and 6-4 photoproducts. In this study, we measured the production of melanin by Fontana Masson staining and the expression of various melanocyte specific proteins, including tyrosinase, TYRP1, DCT, MART1, GP100, and MITF, by immunohistochemical staining in response to UVR. Using these biomarkers, we quantified the proliferation of melanocytes, the expression of melanogenic genes, and the production of melanin in different types of skin. Melanocytes did not proliferate significantly within 1 week after UVR. Levels of melanin were approximately 4-fold higher in darker skin than in lighter skin, and increases in distribution of melanin particles following UVR could be seen after 1 week in all types of skin. Tyrosinase was most effectively stimulated by UVR, and its expression closely mirrored the increases in melanin content in all racial/ethnic groups. The magnitude of the increased tyrosinase expression 1 day after UVR was higher in darker skin type individuals. Expression of MART1 and GP100 was also stimulated by UVR, and their magnitude was higher in lighter skin type individuals. The intensity of TYRP1, DCT, and MITF did not increase remarkably in any racial/ethnic group, but their magnitude was again higher in lighter skin type individuals. We conclude that these biomarkers are useful to investigate the basis of variation in skin color and responses to UV within different racial/ethnic groups.

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Galectin 3 localizes to melanosome membranes in normal human melanocytes

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The mechanisms that regulate melanosome transfer from the melanocyte and its engulfment in the keratinocyte are not fully understood, but are believed to be Rab-dependent and to involve cell surface carbohydrates and their cognate binding lectins. In particular, galactose residues are implicated in the process, since isolated melanocyte vesicular membranes bind galactosyl-linked proteins, and conversely, addition of galactosyl -linked proteins inhibits melanosome transfer to keratinocytes. Since galectin 3 (Gal3) is a lectin that specifically binds galactosyl- glycoproteins, we initiated an investigation of its role in this process. Both human keratinocytes and melanocytes express Gal3 in vivo and in culture. Gal3 is homogenously distributed in the cytosol of cultured keratinocytes, and appears to be associated with vesicular organelles within cultured melanocytes. When melanocytes and keratinocytes are co-cultured, the melanocytes stand out as much more brightly immunostained with antibodies to Gal3. Immunoblot analysis of cell lysates reveals that melanocytes have 5 times more Gal3 than do keratinocytes. Finally, using confocal microscopy, we demonstrate that Gal3 colocalizes with both stage I and II melanosomes (recognized by the polyclonal antibody αPEP1h, against the carboxy terminal of the melanosome specific tyrosinase related protein 1) as well as premelanosomes (recognized by the antibody NK1-beteb against the gp-100 component of the premelanosome and melanosome membrane), present both in the perinuclear and distal dendrite areas of the melanocyte. Thus, we present preliminary evidence of preferential localization of Gal3 in human melanosomes, supporting the hypothesis implicating Gal3 in melanosome recognition and internalization by keratinocytes.

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***AIM-1* gene, OCA4, is one of major loci for Japanese oculocutaneous albinisms**

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Oculocutaneous albinism (OCA) is an autosomal recessive disorder caused by mutations of melanogenic genes such as tyrosinase (OCA1), P protein (OCA2), and DIHCA oxidase (OCA3). In 2001, a novel melanogenic gene named *AIM-1* (AF172849) encoding the membrane-associated transporter protein was identified as the fourth pathological gene in a Turkish patient with OCA, and termed OCA4. At present, no other OCA4 patient has been reported. We have reported that half and 8% of Japanese OCA patients could be classified into OCA1 and OCA2, respectively, but the remaining 33 patients could not be unclassified. In order to clarify the OCA type of 33 patients, we tried to find pathological mutations of the *AIM-1* gene. As a result, seven novel mutations in 17 Japanese OCA patients were detected by PCR-SSCP/heteroduplex screening and direct sequencing of the *AIM-1* gene. These included four missense substitutions (P58S, D157N, G188V, V507L), two deletion mutations (S90del.CG+A91del.CA, V469del.G), and one insertion mutation (V144ins.AAGT). All of the four missense mutations were found at the amino acid residues conserved among medaka fish, mouse, and human. We examined the frequency of seven mutant alleles in 104 unrelated normally-pigmented Japanese subjects (208 alleles), and no allele was detected, indicating that these seven mutant alleles are rare in the Japanese population and can be statistically defined as pathological. These results indicate that *AIM-1* gene, *OCA4*, is one of the major loci for Japanese OCA.

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New strategy for vitiligo treatment: transplantation of genetically-altered melanocytes

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Vitiligo is an acquired pigmentary disorder characterized by white patches of skin due to the absence of pigment-producing cells in the epidermis. Its nature and particular causative remain unknown. Nevertheless, non-surgical and surgical procedures including transplantation of autologous melanocytes have been used in clinical practice. Although successful transplantations of melanocytes have been reported, their survival rate, migration and proliferation potential are unknown. Also the duration of repigmentation after transplantation is questionable, because transplanted melanocytes are fully differentiated and have a limited lifespan. Also in a progressive form of the diseas even surgical approaches are not favorable. Previously we reported simultaneous targeted alteration of the tyrosinase and c-kit genes in albino mouse melanocytes. We attempted to characterize c-kit altered pigment-producing cells in tissue culture conditions and to transplant these cells into the skin of albino animals. Our results indicate that melanocytes with constitutively active Kit receptor do have certain advantages over normal mouse pigment-producing cells: In vitro cells with constitutively activated Kit tyrosine kinase can migrate; proliferation rates of these cells are higher than that of normal melanocytes. In vivo, after transplantation, cells survived in mouse skin for more than 2 months and continue to produce melanin; transplanted, Kit- altered cells, did not possess any tumorigenicity; migration of some cells toward hair follicles and epidermis was also detected. Drawing from these observations, we hypothesize that genetically modified, Kit activated human melanocytes could be used for transplantation via injection for localized treatment of vitiligo and other disorders associated with depigmentation.

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Disruption of the telomere loop induces pigmentation in normal human melanocytes

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Experimental disruption of the telomere loop structure by ectopic expression of a dominant-negative telomere repeat binding factor, $TRF2^{DN}$, induces a DNA damage response leading to apoptosis, cell cycle arrest or senescence, depending on signal intensity and cell type. Normal human melanocytes respond to DNA damage and senescence by increasing their pigmentation, a protective response against further DNA damage. We and others have shown that small DNA oligonucleotides homologous to the telomere 3' overhang also induce these damage responses in melanocytes, in part by transcriptionally upregulating tyrosinase, and hypothesize that exposure of this overhang following telomere disruption by $TRF2^{DN}$, critical shortening during aging or after acute DNA damage is a physiological signal for increased pigmentation. In order to examine the effect of telomere disruption on pigmentation, cultured normal human melanocytes were infected with an adenovirus construct expressing $TRF2^{DN}$ ($AdTRF^{DN}$) or a control virus expressing green fluorescent protein (AdGFP). Microscopic analysis of the AdGFP-infected melanocytes demonstrated an infection efficiency of 90-95%. The pigmentation response was qualitatively and quantitatively assessed by a melanin assay. Compared to melanocytes treated with diluent or AdGFP, those treated with $AdTRF2^{DN}$ decreased their growth rate, became more dendritic and more pigmented as assessed by light microscopy and silver staining, and increased their melanin content over 6 days to 184% of control cells (77 ± 1 vs 42 ± 2 pg/cell), ($p < 0.05$). Finally, we wished to examine the regulatory proteins involved in the pigmentation response. $AdTRF2^{DN}$ specifically increased the protein levels of both tyrosinase and p53, known to regulate tyrosinase, when compared to diluent- or AdGFP-treated melanocytes. In summary, these data demonstrate that experimental disruption of telomere loop structure in cultured normal human melanocytes induces DNA damage responses and suggest that telomere disruption during aging or from exposure to UV light contributes to the tanning response

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19D7 monoclonal antibody identifies immature melanoma cells

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We believe melanocytic neoplasias (benign and malignant) are derived from mutations first occurring in melanocytic/neural crest stem cells. One of our current goals is to develop markers for these early cells. We have identified a melanoma line that has characteristics anticipated for melanocytic precursor/stem-like cells including the capacity to spontaneously give rise to cells with increased proliferative capacity and increased melanin production. We generated monoclonal antibodies against the tumor line and selected for antibodies with increased staining for the immature cell phenotype. The resultant monoclonal antibody, 19D7, was tested against a wide variety of formalin fixed tissue sections of human skin and other organs. It was found to stain a vesicular component of immature melanocytic cells, cells concentrated near nerve and vascular bundles, bone marrow, liver, and the luminal surface of glands. Dermal cells of nevi and melanomas were strongly positive, while melanocytes and melanized tumor cells were largely negative. The antigenic target of 19D7 is between 3 and 10 kDa but also exists in a greater than 50 kDa form. By electron microscopy the antigen appears to be present on the cytoplasmic surface of small vesicular structures. Histologic staining patterns are different than those of HMB-45, TRP-1, DCT, KIT, S100b, and MART. 19D7 appears to identify an antigen present in melanocytic cells prior to their full differentiation and identifies a population of presumptive melanocytic/neural crest stem cells in human skin.

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Tyrosinase-related protein 1 and 2 selectively inhibit cytotoxicity and cell death of melanocytes and melanoma cells occurring in tyrosinase-mediated melanogenesis

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Melanin synthesis by tyrosinase involves the production of cytotoxic free radicals. This study examines the biological function of tyrosinase related proteins (TRP-1 and 2) by using the recombinant adenoviruses that express tyrosinase, TRP-1 or TRP-2. High-level expression of tyrosinase introduced not only the synthesis of new melanin but also the cell death in both of the melanocytic and non-melanocytic cells. This cell death was found to be non-apoptotic through FACS analysis. When TRP-1 or TRP-2 was co-expressed with tyrosinase, this cell death was significantly reduced in melanocytic cells, but not in non-melanocytic cells. Western blot analysis revealed that TRP-1 does not affect the amount of co-introduced tyrosinase and TRP-2, the amount of melanin and/or eumelanin (not pheomelanin) increased substantially more than that in cells expressing tyrosinase alone. By double immunostaining of tyrosinase and TRP-1, the two proteins were co-localized in melanocytic cells but not in non-melanocytic cells. Our findings indicate that TRPs play an essential role in inhibiting tyrosinase-mediated cytotoxicity in melanocytic cells, possibly through the compartmentalization of new melanogenesis in melanosomes without altering melanin production.

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Phenotypic expression of melanocortin-1 receptor mutations in Black Jamaicans

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The melanocortin 1 receptor (MC1R) is the only gene identified that explains substantial variation in hair colour. Although there has been interest in studying the evolution of the MC1R, genotype/phenotype relationships outwith European populations are limited. We recently studied persons with red hair but who self identify themselves as Black. In Jamaica there are persons who self-identify themselves as Black who have auburn/reddish hair, freckles, and a 'rust-coloured' complexion (sometimes called 'red Ibos'). As part of an investigation of the phenotypic expression of MC1R variants in a Black population we have examined MC1R sequence and hair melanins in 4 Jamaican'redheads'. Samples were obtained from four redheads (ages 3, 11, 30 and 32 years; 2 boys and 2 women respectively) and also from the mothers of the 2 boys. Sequencing of MC1R revealed that all of the redheads were compound heterozygotes for variants that are either known, or predicted to disrupt MC1R function. MC1R genotypes were as follows (variant positions compared to human consensus sequence: GenBanK X65634): subject #1: Gly89Arg/Arg151Cys (his mother is heterozygous for the Arg151Cys variant); subject #2: 23 STOP/Arg160Trp (his mother is heterozygous for the 23 STOP variant); subject #3: Asp294His/frameshift at codon 195; subject #4: 23 STOP/Arg151Cys. Hair melanins for subjects #1, #2 and #3 were as follows: log eumelanin 8.3, 8.3, 7.6; log pheomelanin 4.2, 6.6, 6.5. All of these values are within the range of values seen in a white UK sample of MC1R homozygotes selected on the basis of the red hair phenotype (log eumelanin mean 7.5 (standard deviation, SD 0.6); log pheomelanin 6 (SD 0.9).

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B1,6-branched N-glycans and coarse vesicles in melanoma progression

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B1,6-branched N-glycans, catalyzed by GNT-V, predict poor survival in animal and human cancer, yet little is known on a histopathologic level. Is upregulated GNT-V associated with a cellular phenotype? We studied B1,6-branching and cell morphology in 57 human melanomas (18 in situ, 26 vertical phase, 13 metastatic), and 60 primary tumors of diverse origin. Melanin was decolorized and sections stained with the lectin LPHA (B1,6-branched N-glycans) and anti-CD63. Both stains co-localized to vesicular structures, which, in melanized regions of melanomas, were present as coarse melanin. Similar LPHA-positive vesicles but lacking melanin were found in amelanotic tumor cells. When melanomas were scored for % LPHA-positive, vesicular cells/section, primary tumors were heterogeneous, ranging from 0-100% of the cells being positive. Using micro-tissue array, LPHA-positive vesicles were also observed in 55 of 60 primary tumors of diverse origin, including carcinomas of the lung, breast, prostate, and colon, again with LPHA staining from 0-100% of tumor cells. In contrast, melanoma metastases were more homogeneous, with vesicular, LPHA-positive cells comprising 75-100% of each section examined. Increased LPHA positivity in metastases vs primary tumors was significant (p=.0080). Thus, a study of LPHA staining in melanomas and other tumors revealed a previously unrecognized phenotype: co-expression of cytoplasmic coarse vesicles and B1,6-branched N-glycans. This phenotype was detected in primary tumors, but was most prominent in metastases. A recent report from others showed that GNT-V, when transfected into mink lung cells, elicited formation of LPHA-positive vesicles (Hariri et al., Mol Biol Cell, 11:255-268 2000). Thus, it is possible that the vesicles in tumors here were induced by elevated GNT-V. Our findings are also in line with known correlations between increased GNT-V expression and metastasis, and as such could provide a new understanding of the biology of this process, particularly regarding the role of B1,6-branching and aberrant N-glycosylation.

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Chemical genetic approach to mammalian pigmentation

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Chemical genetics is an approach used to investigate cellular pathways and to identify novel gene products by conditionally modulating protein function through the use of small molecules. This can be achieved with remarkable specificity by designing large and structurally diverse chemical libraries that are subsequently screened for a desired cellular phenotype. The purpose of our investigation was to identify a new class of pigment enhancing compounds from a triazine based chemical library with the intention of utilizing these molecules bound to an affinity matrix to study proteins involved in pigmentation. We report the synthesis and screening of an initial 1,600 novel small molecules and the identification of a class of similar chemicals that augment pigmentation in cultured melan-A melanocytes. Active compounds were structurally derivatized to maximize their cellular effectiveness. One molecule enhanced pigmentation by as much as 500% at a concentration of 5uM. This new class of chemicals increases melanin production, tyrosinase activity and tyrosinase, TRP-1, TRP-2, protein levels in a dose-dependent manner at nontoxic effective concentrations. Treating melanocytes with these chemicals in combination with the known promoter IBMX results in a dramatic increase in melanin production, tyrosinase activity and tyrosinase, TRP-1, TRP-2, protein levels. Phenotypic analysis of treated cells suggests that the active compounds and IBMX enhance pigmentation by different mechanisms. The most active lead molecules have been used to create agarose bead affinity matrices for protein target isolation. In conclusion we demonstrate that applying a chemical genetic approach to pigmentation yields a valuable class of reagents that have been structurally manipulated to maximize their cellular effects and to facilitate protein target identification. We believe these chemicals work to enhance melanin production by molecular mechanisms distinct from those stimulated by IBMX.

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Characterization of the molecular defects in RAB27A, caused by RAB27A missense mutations found in patients with Griscelli syndrome

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Griscelli syndrome (GS) is a rare genodermatosis, characterized by an accumulation of melanosomes in melanocytes resulting in pigmentary dilution. Mutations of the myosin-Va and Rab27a genes have been identified in GS patients. On the other hand, mutations of the melanophilin gene account for the pigmentary dilution observed in the leaden mouse. It has been shown that melanophilin, Rab27a and myosin-Va constitute a tripartite complex that controls melanosome distal transport and dendrite targeting. Interestingly, three GS patients present single homozygous, missense mutations, W73G, L130P and A152P. First, we studied the effect of overexpression of these mutants on melanosome, melanophilin and myosin-Va localization in B16 melanoma cells. Then, we evaluated several key parameters for Rab27a function, including GTP binding activity, interaction with melanophilin and with myosin-Va. Our results showed that Rab27a-L130P cannot bind GTP and consequently cannot interact with melanophilin. Rab27a-L130P acts as dominant-negative mutant since its overexpression alters melanosome, melanophilin and myosin-Va localization in B16 cells. Interestingly, Rab27a-W73G binds GTP, but does not interact with melanophilin. Rab27a-W73G does not interfere with endogenous Rab27a and does not impair melanosome transport when overexpressed in B16 cells. In the GS patient with homozygous W73G mutation, impairment of melanosome transport can be explained by the exclusive expression of Rab27a-W73G. Finally, Rab27a-A152P binds both GTP and melanophilin. However Rab27a-A152P acts as a dominant negative mutant since its overexpression, in B16 melanoma cells, blocks melanosome transport and mimics a GS phenotype. We have not been able to identify the molecular defect in Rab27a-A152P, but our results point out to the importance of the Alanine 152 in a Rab27a function other than GTP binding or melanophilin interaction.

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Genetic determinants of dermal and epidermal melanocytosis

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Melanocytes undergo a series of genetically controlled behaviors during development as they migrate from the neural crest, through the embryonic dermis, and finally to specialized structures in the epidermis. Some of the molecular pathways involved in these processes have been identified and studied using mutations that cause white spotting. Recently, however, large-scale mutagenesis projects in mice have enabled us to use forward genetics to study a novel class of mouse pigmentation mutants identified by dark skin (*Dsk*). Among ten dominant Dsk mutants, we have identified three, *Dsk1*, *7*, and *10*, that cause an abnormal expansion of pigment cell precursors very early in development, and one, *Dsk3*, that causes an expansion of pigment cell number that becomes apparent 2 - 3 weeks after birth. The former class of mutations leads to an accumulation of dermal melanocytes, but the latter causes accumulation of epidermal melanocytes. Detailed characterization reveals the existence of a mechanism for limiting the number of pigment cells allowed into the developing epidermis. We identified the molecular lesions responsible for *Dsk1*, *7*, and *10* as missense alterations in *Gnaq* or *Gna11* that likely represent a gain-of-function, leading to increased phosphatidylinositol-3 kinase signaling and abnormal melanoblast proliferation. The *Dsk1*, *7*, and *10* mutations are semidominant and additive, but *Dsk3/Dsk3* mutant animals die early in embryonic development. Thus, for both classes of mutations, genetic lesions identified by virtue of their effects on pigmentation provide entry points for studying fundamental aspects of cell signaling and cell biology.

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Rapid quantitation of immunostained cultured cells in a formalin-fixed, paraffin-embedded fibrin cell block

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Our laboratory routinely suspends cultured human melanocytes in a fibrin cell block (FCB) followed by formalin-fixation and paraffin-embedding. Our laboratory utilizes FCBs for confirmation of cell lineage and estimation of population purity of cultured human melanocytes by immunostaining with specific melanocyte markers such as Melan-A. This technique also provides a permanent archive of cultured cells for future studies. The objective of this study was to develop an image analysis protocol designed to determine the percentage of cells labeling with various immunohistochemical markers using the alkaline phosphatase substrate, naphthol/Fast-Red, the red product of which is stable, does not fade with storage, and is autofluorescent. The autofluorescence of Fast- Red is especially useful when the paraffin-embedded FCB slices are counterstained with 4', 6-diamidino-2-phenylindole (DAPI) which forms fluorescent complexes with double-stranded DNA and therefore stains nuclei. Accordingly, the total number of cell nuclei can be visualized with excitation wavelength of 350 nm. Immunofluorescence of Fast-Red labeled cells can be visualized with excitation wavelength of 510 nm. Low power (100x) images are digitally photographed with both 350 nm and 510 nm excitation wavelengths to identify the total number of cells (DAPI) and those labeling the antigenic marker of interest (Fast-Red). The digital images are analyzed and cells are counted with NIH Image 1.63. Up to four sequential fields are photographed in this manner, each containing several hundred to more than one thousand cells per field. Thus, several thousand cells can be counted easily and quickly and the percentage of immunolabeled cells can be derived. Preliminary experiments suggest that this technique can be used for cell cycle analysis using immunohistochemical markers such as topoisomerase II and MIB-1.

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Enhanced Erk phosphorylation and melanogenic enzyme expression in *Nf1* +/- melanocytes

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The role of the Ras-GTPase activating protein neurofibromin, encoded by the *Nf1/NF1* genes, in mediating both Kit-independent and Kit-dependent Erk activation in murine melanocytes was examined in this study. Prior work has shown that neurofibromin haploinsufficency causes partial reversion of a coat color deficiency in Kit^{W41}/Kit^{W41} mice and is associated with characteristic pigmentary (café-au-lait) lesions in patients with type I neurofibromatosis (NF-1). KIT-mediated signaling is known to induce Ras-dependent phosphorylation of the MAP kinase Erk-2 and the melanocyte transcription factor MITF. We thus examined both MAP kinase activation and melanogenic enzyme expression in neurofibromin-deficient melanocytes. *Nf1* +/- and +/+ primary melanocytes were successfully cultured from dermal suspensions of wild-type and *Nf1* heterozygous knockout littermates. Cells were stimulated with the Kit receptor ligand stem cell factor (SCF) for 0 min and 15 min. Cell lysates were probed for expression of Erk-2, phospho-Erk (P-Erk), MITF, and melanogenic enzyme expression by Western analysis. Primary cultures of *Nf1* +/- melanocytes exhibit similar morphology to wild-type cells when cultured up to 60 days. *Nf1* +/- cells exhibit enhanced expression of P-Erk prior to Kit stimulation. Kit stimulation increases P-Erk preferentially in *Nf1* +/- cells following Kit stimulation in comparison to wild-type cells. Both Kit-independent and Kit-dependent increases in P-Erk in *Nf1* +/- cells are inhibited by incubation with the Mek inhibitor PD98059. Expression of the melanogenic enzymes tyrosinase, TRP-1, and Dct/TRP-2 are also increased in *Nf1* +/- melanocytes. These results show that P-Erk is increased in *Nf1* +/- melanocytes independent of Kit stimulation, and that neurofibromin haploinsufficiency potentiates the effect of Kit stimulation on MAP kinase activity. Increased expression of melanogenic enzymes in *Nf1* +/- melanocytes may be a determinant of the appearance of café-au-lait macules and patches in NF-1 patients.

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Identification of antigenic markers with increased expression in immature melanocytic cells

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We believe that melanocytic precursor/stem cells are the initial target of the mutagenic events leading to melanoma/nevus development. The purpose of this study was to identify markers for early melanocytic differentiation states. Utilizing a melanoma line that differentiates from a precursor phenotype to a more differentiated phenotype we performed an Affymetrix U95A microarray analysis of both forms and identified genes highly expressed in the immature form that were down regulated with differentiation. This list was then matched for commercially available antibodies to determine whether the antigens were also down regulated. The antibodies matching genes that were down regulated included: glucose transporter-like protein-III (GLUT-3), N-RAS, histone H2B, c-MYC, metallothionein-I-A, HLA DR, E-cadherin, and secretogranin II. Studies against the immature and differentiated forms of the tumor line confirmed that N-RAS, HLA DR, secretogranin II, H2B, GLUT-3, and c-MYC were down regulated with differentiation. The results with E-cadherin and metallothionein-I-A were inconsistent. The antibodies for HLA DR, H2B, GLUT-3, and secretogranin II also worked in formalin-fixed paraffin-embedded tissue and positively stained foci of nevi/melanoma cells but failed to reveal significant staining of the epidermal melanocytes. We conclude that the expression of these markers in melanocytic cells identifies cells that have not yet completed the differentiation process.

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Immature dermal melanocytic cells are present in growing melanocytic neoplasms

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The objective of this study was to describe histopathologic characteristics of growing melanocytic neoplasms. Clinical photos and tissue obtained from pigmented lesion clinic patients were retrospectively examined. Lesions were classified as changing if there was at least 2 out of 3 agreement of greater than two-fold enlargement on photos by three dermatologists masked to the identity and history of the patient. IRB approved protocol allowed the use of tissue sections from the surface of the residual neoplasm in the block, thus only a portion of each of these lesions was available for study. The tissue sections were stained with H&E and 19D7 (an antibody that identifies immature melanocytic cells). Selected slides were also stained with S100b and Ki-67. Histopathological evaluation revealed that 58% (34/59) of the specimens included melanocytic cells in the papillary dermis. Large junctional nests (>75% of epidermal thickness) were common (25/59) and expulsion of some of these nests through the epidermis was noted. Upwardly migrating cells were also common (19/59). 19D7 labeled the dermal cells in the majority of lesions. S100b also stained these cells. Ki-67 positivity was rare but could be identified in the dermal component including one case in which only a few cells at the base of the dermal component were found to be positive. Thus, our data suggests that in the majority of acquired melanocytic neoplasms (benign and malignant) a papillary dermal component is present during the growth phase and this component is 19D7 positive. In addition, some epidermal nests and individual melanocytic cells appear to be extruded through the epidermis. We propose that many acquired melanocytic neoplasms may originate in an immature population of cells in the papillary dermis with cells migrating into the epidermis where they accumulate and may also be extruded. Cells not able to migrate effectively into the epidermis accumulate in the dermis as this process continues.

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Foxn1 and the acquisition of pigmentation

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Foxn1 (Whn, Hfh11) is a transcription factor containing a winged-helix DNA-binding domain and a negatively charged transactivation domain. In rodents, the loss of Foxn1 function causes the nude phenotype, which is characterized by the lack of visible hair, structural abnormalities in the epidermis, defects in lactation, and the absence of a thymus. To assess the effects of a gain of Foxn1 function, we generated transgenic mice that express *Foxn1* from the keratin 5 (Krt5) promoter. This promoter targeted Foxn1 to basal epithelial cells of the epidermis and hair follicles, cell types generally lacking endogenous Foxn1. Surprisingly, the *Krt5-Foxn1* transgene caused changes in skin pigmentation. In contrast to wild-type epidermis, the transgenic epidermis contained melanin, and the basal layer exhibited numerous melanocytes. While maintaining this epidermal melanocyte population, the transgenics showed no abnormalities in coat color or density, suggesting that melanocytes entered the hair follicles in normal numbers. Thus, the transgene reshaped the pigmentary system and made it more human-like, as the system provided pigment to both the epidermis and hair. In wild-type hair follicles, Foxn1 was most abundant in the differentiating precursors of the cortex, which receive pigment from melanocytes; to a lesser extent, Foxn1 was also detected in the differentiating medulla, the other melanized structure of the hair. Since Foxn1 is normally found in melanocyte targets, we propose that Foxn1 promotes the functional association of melanocytes and epithelial cells. In all, these results provide the first evidence of a role for Foxn1 in pigmentation.

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Selective glucocorticoid receptor agonists express an improved effect/side effect profile *in vivo*

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Glucocorticoids (GCs) represent a well known class of anti-inflammatory and immunosuppressive drugs. They are used in the treatment of many chronic inflammatory and allergic diseases. However, their therapeutic effects are often accompanied by the development of severe and sometimes irreversible side effects. Thus, there is a real need for "better" GCs, meaning GCs with a reduced side effect induction while maintaining the anti-inflammatory and immunosuppressive properties of classical GCs. GCs affect gene expression by either a transactivation or a transrepression mechanism. It has been accepted, that anti-inflammatory effects are mainly mediated via the transrepression mechanism, while many side effects are induced by the transactivation mechanism. Therefore, our aim was the identification of glucocorticoid receptor (GR) ligands, that preferentially induce transrepression and if at all a weaker transactivation. Here we show a novel non-steroidal compound class with a significant dissociation of transactivation from transrepression in vitro and in vivo. In vitro a preference for the transrepression mechanism was found and cytokine expression in human PBMCs could be inhibited. Both systemic and topical applications of a representative of the new compound class indicated that it has anti-inflammatory activity similar to prednisolone. In rodents we were able to demonstrate superiority of the SEGRA compound over prednisolone with regard to blood glucose induction, spleen involution and to a lesser extent skin atrophy but not to ACTH suppression. Thus, a dissociation of transactivation and transrepression mechanisms in vitro is indeed reflected by a reduction of side effects in vivo. SEGRAs represent promising new drug candidates with an improved effect / side effect profile since they should have a lower risk for transactivation mediated side effects, e.g. diabetes mellitus.

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Androgen metabolizing activity of retinol dehydrogenase-4 (RoDH-4) is inhibited by 13-*cis*-retinoic acid (isotretinoin) but not by all-*trans* retinoic acid

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Severe acne is efficiently treated by systemic 13-*cis*-retinoic acid (isotretinoin). The mechanism of action of 13-*cis*-retinoic acid has been an enigma since it does not bind to the nuclear retinoic acid receptors (RARs). Although, a specific conversion of 13-*cis*-retinoic acid into all-*trans* retinoic acid was recently found in cultured sebocytes, additional effects not mediated by RARs might exist. An enzyme expressed in human epidermis, RoDH-4, is oxidizing retinol and 13-*cis*-retinol into corresponding aldehydes but also 3-α-androstandiol into dihydrotestosterone and androsterone into androstandione. Of special interest for acne is the production of dihydrotestosterone by this back-reaction. Here we examined the inhibitory effect of several natural and synthetic retinoids on the androgen metabolizing activity of microsomes prepared from Sf9 insect cells over-expressing recombinant RoDH-4. We found a strong competitive inhibition by 13-*cis*-retinoic acid but less by other natural and synthetic retinoids, i.e. all-*trans* retinoic acid, acitretin and adapalene. However, two other natural retinol metabolites known to be present in human epidermis and sebaceous glands; 3,4-didehydroretinol and 3,4-didehydroretinoic acid, inhibited the androgen metabolizing effect of RoDH-4, albeit 2-10 fold less efficient than by 13-*cis*-retinoic acid. Thus, 13-*cis*-retinoic acid possesses unique properties unrelated to retinoid receptor activation which might in part explain the mechanism of action of this drug in acne.

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Combination of topical calcipotriol and the narrow band UVB in the treatment of vitiligo

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Vitiligo is a common condition affecting 1-2% of general population. PUVA and narrow band UVB (NB-UVB) have been shown to be effective treatments. It has been postulated that there was a defective in calcium homeostasis in affected skin; therefore calcipotriol, a vitamin D3 derivative, may also have a beneficial role in vitiligo. Previous studies have shown that calcipotriol enhanced the effectiveness of PUVA in treatment of vitiligo. The purpose of this study was to determine the efficacy of topical calcipotriol / NB-UVB combination, compared to NB-UVB alone in the treatment of vitiligo. Twenty patients with bilateral symmetrical vitiligo affecting •5%-50% body surface areas were enrolled in the study; study period was August 2001 to October 2002. Patients were exposed to NB-UVB three times a week, starting with 280 mJ/cm2, and dose was increased by 10-15% per treatment. Topical calcipotriol was applied to all lesions on the left side of the body twice daily. Seventeen patients (11 males and 6 females, age range: 14-68 years) completed the study; 3 patients failed to properly follow the instruction for application of calcipotriol. Of the 17 patients, there were 8 African-Americans, 8 Caucasians, and 1 Hispanic. All patients tolerated the combination treatment well. Intent-to-treat analysis was used. The overall response is as follow: 8 patients(8/20=40%) had significant(66-100%) repigmentation, 6 patients(30%) had moderate(25-65%), 1 patient(5%) had mild(10-24%), and 2 patients(10%) had minimal(less than 10%) improvement. Nine patients(3 patients, 5 patients and 1 patient with significant, moderate and minimal improvement, respectively) noticed appreciable improvement on the left side by 29-114 treatments. As treatment was continued, the response equalized on both sides in 3 patients. Lesions on trunk responsed better than those on hand and extremities. In conclusion, topical calcipotriol / NB-UVB combination is a therapeutic option that result in a more rapid pigmentation in some patients with vitiligo.

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The mechanism promoting skin wound healing through the topical application of sugar and povidone-iodine

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A combination of 70% sugar and 3% povidone-iodine with an improved vehicle (UP) has been used to accelerate granulation tissue formation in patients with refractory cutaneous ulcers and pressure sores. However, the healing mechanism has remained unclear. The present study examines changes in cytokine production, extracellular matrix expression and macrophage immigration by means applying UP, sugar or povidone iodine to cultured human fibroblasts and cutaneous full-thickness wounds in mice. A high concentration of UP depressed the proliferation of fibroblasts. On the other hand, it accelerated collagen synthesis in cultured fibroblasts and increased TGF-beta 1 concentration in wound tissues. These effects were induced by sugar. Furthermore, UP accelerated not only the expression of beta 1 integrins in both the cultured fibroblasts and wound tissues, but also increased the immigration of macrophages into wound tissues, effects which were induced by povidone iodine. The results obtained suggested that the application of UP to wounds enhances not only collagen synthesis and ECM expression in cells, but also the migration of fibroblasts and macrophages through the combined effect of sugar and povidone iodine, leading to the acceleration of wound healing.

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Photodegraded nifedipine increases iron in mouse skin

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Epidermal desquamation might be a way of treating iron overload, if more than the normal amount of systemic iron could be delivered to the epidermis. Previous work has demonstrated that photodegraded nifedipine (PDN) increases iron transport into cultured keratinocytes 80-fold, and increases iron content in stratified keratinocyte cultures by 3-fold. We tested whether PDN would increase iron in epidermis following topical application in vivo. The active ingredient in PDN, the nitroso-derivative of nifedipine, was recrystallized from solution following exposure of nifedipine to fluorescent light. Solutions of this nitroso-derivative were applied repeatedly to mouse skin using several different protocols, and iron content was measured following heat separation of epidermis from dermis. Baseline iron content in epidermis was significantly different in different strains of mice. PDN consistently increased iron in epidermis, but never more than 2-fold. Results were similar in ear and flank skin. Twice daily application was better than daily application. Topical application of iron ionophores, such as PDN, may load sufficient iron into desquamating epidermis to have a systemic effect.

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Effects of two topical antimicrobial agents on *Staphylococcus aureus* biofilms in a porcine model

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Biofilms are a combination of microorganisms and extrapolysaccharide matrices. Our laboratory has previously demonstrated that biofilms are present in both acute and chronic wounds. Once biofilms are established, phagocytosis and diffusion of antibiotics are impaired thus contributing to antimicrobial resistance due to increased bacterial virulence. The purpose of this study was to determine the efficacy of two topical antimicrobial agents on partial thickness wounds containing Staphylococcus aureus biofilms. All wounds were inoculated with 106 CFU/ml and covered for 48 hrs under a polyurthene film to promote biofilm formation. Wounds were divided into three treatment groups; triple antibiotic ointment (Polymyxin B sulfate, bacitracin zinc, neomycin), mupirocin cream and untreated control. Wounds were treated twice daily. Wounds were cultured for bacterial quantitation at 24, 48, 72, 96, and 120 hrs. Significant reduction in CFU/ml was observed only after several days of treatments. This finding supports the antimicrobial resistance that occurs when bacteria live within biofilms. Our previous studies demonstrated that both of these agents were able to completely eliminate planktonic S. aureus (106) at the early time points. This study demonstrates that when bacterial biofilms are established in wounds there is a longer response time for topical antimicrobial activity suggesting bacterial resistance.

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Topical oxygen therapy

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A simple method of local oxygen delivery to wounds may have important implications during wound healing. Oxygen is a required substance for various aspects of wound repair and increased oxygen tension in a wound has been shown to stimulate phagocytosis and speed the degradation of necrotic wound tissue. An increase in oxygen tension has been shown to enhance collagen production, neovascularization and neutrophil mediated oxidative microbial killing. We describe here the investigation of a topical aqueous oxygen emulsion (AOE), which is a patented technology based on a supersaturated oxygen suspension using perflurocarbon components. Second-degree burn wounds were created on the backs of specific pathogen free pigs. Burn wounds were then randomly assigned to one of the following treatment groups: AOE, AOE vehicle, or air exposed control. The topical AOE was able to significantly enhance the rate of epithelization as compared to both vehicle and untreated control. This data suggests that topical oxygen is beneficial for epithelization of ischemic wounds such as burns and additional studies examining the potential use of this therapy on chronic wounds is warranted.

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Immunological consequence of pharmacological disruption of endogenous redox regulatory pathways

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The thioredoxin and glutaredoxin systems control many cellular activities by maintaining cytoplasmic proteins in their reduced state. To determine the immunological consequence of an excessively lowered redox potential, we added a selenium-containing anti-oxidant compound PZ51 to the *in vitro* Ag presentation systems composed of: a) a stable dendritic cell (DC) line XS52, KLH-specific Th1 clone HDK-1, and KLH, or b) bone marrow-derived DC, naive or Th1/Th2-primed CD4 T cells from DO11.10 transgenic mice, and OVA peptide. PZ51 inhibited Ag-specific, DC-induced proliferation and production of IL-2, IL-4, and/or IFNγ by each tested T cell population, with 30-100% inhibition achieved at 5-15 μM. PZ51 also suppressed T cell-dependent production of IL-6, IL-12, and TNFα by DC in the same dose range. Unlike cyclosporin A, rapamycin, and dexamethasone, which differentially inhibited selected aspects in Ag presentation, PZ51 inhibited all tested events accompanying bi-directional DC-T cell interaction in an extremely narrow dose range. LPS treatment significantly ($P<0.05$) altered the expression of 223 genes in bone marrow DC, and PZ51 differed from the conventional immunosuppressants in its relative inability to reverse DC expression of this signature gene profile. Allergic contact hypersensitivity (CH) responses of BALB/c mice to oxazolone were suppressed significantly (>50% inhibition and $P<0.001$ in four experiments) by post-challenge treatment with oral administrations of PZ51 (100 mg/kg/day), the protocol causing rapid elevation (<2 hr) and continuous maintenance (>24 hr) of serum PZ51 levels above the *in vitro* identified immunosuppressive dose (15 μM). By contrast, PZ51 failed to inhibit irritant CH responses to croton oil, indicating disease specificity of its action. These results introduce a new concept that redox regulation plays a key role in DC-T cell communication during Ag presentation. Our data also suggest that PZ51 and other anti-oxidants may be used for the treatment of immunological disorders.

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Analysis of intravenous immunoglobulin for the treatment of toxic epidermal necrolysis using SCORTEN: the University of Miami experience

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Toxic epidermal necrolysis (TEN) is a rare, life-threatening condition caused by certain medications. Keratinocytes affected by TEN have been found to undergo apoptosis mediated by Fas-FasL interactions. Intravenous Immunoglobulin (IVIG) has been proposed to inhibit this interaction. SCORTEN system, a validated predictor of TEN mortality, was used to retrospectively analyze the data of consecutive patients diagnosed with TEN treated with IVIG at the University of Miami in order to determine the effect of IVIG on patient mortality. Sixteen consecutive patients admitted as inpatients to the dermatology service at the University of Miami were treated with IVIG at 1g/kg/d for 4 days (total 4g/kg). For each patient, the 7 independent risk factors [age >40 years, presence of malignancy, %BSA involved >10%, BUN >28mg/dL (>10mmol/L), glucose >252mg/dL (>14mmol/L), HCO3 <20mEq/L (<20mmol/L), and heart rate >120 bpm within the first 24 hours of admission] of SCORTEN prior to IVIG therapy were recorded, as was cause of TEN and other medical problems. One patient died. Based on the SCORTEN system, 5.81 patients were expected to die. These mortality rates were compared using the Standardized Mortality Ratio analysis (SMR) to determine the efficacy of this treatment. TEN patients treated with IVIG had a 83% reduction in mortality (95% CI: 0.0-0.96). Our data supports the use of IVIG for the treatment of TEN patients. Based on comparison of our mortality rate to the SCORTEN predicted mortality rate, treatment with IVIG was shown to significantly decrease mortality in TEN patients.

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PUVA vs. PUVA and interferon alpha for treatment of mycosis fungoides

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In the early stages of mycosis fungoides (MF), PUVA has been demonstrated to be an effective disease-modifying therapy. Within the past two decades, the combination of PUVA + interferon alpha (INFa) therapy has been used with promising results. To summarize over ten years experience in the management of MF combining our experience from both a retrospective and a prospective series to evaluate efficacy of PUVA +/- systemic IFNa in treating early MF. A total of 48 patients were examined. In the retrospective chart review, there were 22 males and 15 females, age 22 to 81 (median 53 years). 17 subjects were African-American and twenty were Caucasian. In the randomized prospective trial, there were 9 males and 2 females, age 37 to 76 (median 64 years). Nine of these patients were Caucasian, 1 was African-American, and 1 was Asian. Of the total 48 patients, 36 (12 females and 24 males) were treated with PUVA alone and 12 (5 females and 7 males) received PUVA + INFa therapy. In the retrospective chart review, a complete(CR) or partial response(PR) was achieved in 28/30 subjects in the PUVA alone group(93.0%), while in the PUVA + INFa group a CR or PR was attained in 5/7 patients(71.0%). In the randomized arm, 5/6 patients from the PUVA alone group (83.0%) achieved CR or PR, and 5/5 patients (100%) from the PUVA + INFa group attained CR or PR. Our study showed no significant difference in the outcome of the PUVA alone treatment group vs. PUVA + INFa combined treatment group.

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Gene expression in actinic keratoses—pharmacological modulation by imiquimod

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Imiquimod, an imidazoquinoline amine, is an immune response modifier approved for the topical treatment of external genital warts. Preliminary studies showed antitumorous effects. In vitro studies showed that imiquimod stimulates the synthesis of IFN-α, IFN-γ, IL-1, -6, -8, -12 and TNF-α. However, the mechanisms of imiquimod's activity are not clearly understood. Our aim was to study the clinical and molecular effects of topical imiquimod in the treatment of AK. 13 patients with AK were treated with imiquimod 5% cream over night 3 times per week for 6 weeks. Shave biopsies were taken from healthy skin, AK lesional skin before treatment, during the treatment at the peak of the inflammation and 2-3 weeks after the treatment. Generally we observed a positive response to imiquimod. 12 patients showed a total or almost complete clearence of the treated lesions, 1 patient was a non-responder. To gain insight into the molecular processes induced by imiquimod, we investigated the expression of several genes coding for inflammatory cytokines (IL-6, TNF-α, IFN-α), IL-10R, antiapoptotic proteins (Hurpin, Hax-1), proteins involved in cell-adhesion (α- and β-catenin) and the tumor suppressor gene p53. Additionally, we analyzed the expression of TLR-7, possibly mediating the activity of imiquimod. Total RNA was isolated from biopsies and gene expression was analyzed by semiquantitative RT-PCR. We found a significant upregulation of the proinflammatory cytokines IFN-α, IL-6 and TNF-α which correlates well with the local inflammation induced by imiquimod. Interestingly, the transcription of IL-10R and TLR-7 was also significantly increased during imiquimod treatment. In contrast, expression of Hax-1, Hurpin, p53, α- and β-catenin was not significantly influenced by imiquimod. Effects of imiquimod in AK correlate with specific influences on gene expression.

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Anti-infammatory activity of topical nanocrystalline silver cream on allergic contact dermatitis in a guinea pig model

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The objective of the study was to assess the anti-inflammatory activity of nanocrystalline silver cream and compare the effects with those of tacrolimus ointment (Protopic 0.1 %), using a guinea pig model of allergic contact dermatitis. Dermatitis was induced using 5% dinitrochlorobenzene. Once dermatitis was established, nanocrystalline silver 1% cream, tacrolimus ointment, or appropriate control formulations was applied topically once daily for 5 days to groups of 10 animals each and compared to animals receiving no treatment. Erythema and edema were evaluated daily (on a score of 0 to 4, from absent to very severe/excessive) and by histopathology of skin biopsies after five days of treatment. Prior to the onset of treatment, erythema and edema scores averaged 3.8 to 3.9 and 2.9 to 3.3, respectively. In the no treatment group these scores remained at about this level for the subsequent 5 days of the study. The respective control groups for each of the active treatments did not differ significantly ($P>0.05$) from the no treatment group. In contrast, within one day of treatment 1% nanocrystalline silver cream reduced erythema and edema significantly ($P<0.001$) and this reduction progressed throughout the study period so that after five days of topical treatment, mean scores reached 1.2 and 0.5 for erythema and edema, respectively. Tacrolimus produced similar decreases in erythema and edema, with no significant differences compared to nanocrystalline silver. Skin biopsies were also evaluated on a 0-4+ scale for degree of inflammatory response. Control treatments could not be differentiated from no treatment group. Nanocrystalline silver and tacrolimus markedly decreased inflammatory scores ($P<0.001$), but were not different from each other. This study suggests that nanocrystalline silver cream has therapeutic potential for topical treatment of inflammatory skin diseases.

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Development of novel NFκB decoy oligonucleotide ointment and the first successful clinical trial for severe atopic dermatitis patients

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NFκB is a transcriptional factor regulating expression of various inflammatory genes. In this study, we have developed novel anti-inflammatory ointment containing NFκB decoy oligodeoxy nucleotides (NDON) which bind NFκB to neutralize its transcriptional activity. Topical application of 1.5% NDON ointment to the NC/Nga atopy model mice skin resulted in a marked improvement of the dermatitis, accompanied by a significant decrease of mast cell infiltration because of their apoptosis. Number of ICAM1-positive cells was also significantly decreased in the NDON-treated skin. Based on those observations, we have designed a first clinical NDON trial for treatment of severe atopic dermatitis. Prior to the trial, adult volunteer patients were treated by vehicle ointment only for two weeks. Finally, 7 volunteer patients, whose dermatitis conditions were not changed or the worse, agreed to enter the trial. The first week of treatment by one-time NDON topical application per day was followed by intermittence for the second week, and the third week of NDON treatment by two-time administration per day was again intermitted for the fourth week before final evaluation. Severity and improvement of the skin involvement, local and systemic side effects and score of pruritis were evaluated once a week. After the trial, six out of the 7 patients revealed significant improvement, especially for severe facial involvement, although lesser effect was observed on the lesion of the trunk and the extremities, presumably due to low permeability of NDON on these regions. No local and systemic side effect was observed. Noteworthy points of NDON treatment were observations of no rebound and of prolonged improvement without potent steroid administration after NDON termination, suggesting quite unique action mechanisms involving mast cell apoptosis. These successful results promise NDON as a novel therapeutics especially for severe facial atopic dermatitis.

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The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis in cutaneous T cell lymphoma cells

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Mycosis fungoides (MF), an indolent cutaneous T-cell lymphoma (CTCL) characterized by accumulation of epidermotropic CD4+CD45RO+ helper/memory T cells in skin, may result from loss of normal, activation-induced apoptosis. Pharmacological modulation of apoptosis may provide novel therapeutic approaches for CTCL. Suberoylanilide hydroxamic acid (SAHA), a potent inhibitor of histone deacetylases (HDACs), causes growth arrest, differentiation, and/or apoptosis of several types of tumor cells both in vitro and in vivo. SAHA is in clinical trials for CTCL thus we wanted to determine its effect on cell lines and patients's cells. Human CTCL cell lines (Hut78 and HH) and peripheral blood lymphocytes (PBLs) from 6 CTCL patients at baseline were treated with 0.1, 0.5, 1, 2.5, 5, and 10 uM SAHA for 24 and 48 hrs. Apoptosis was assessed by annexin V binding and cell cycle arrest measured by flow cytometry, and proteins by Western blotting. In HH cells, apoptotic rates of 43-70% over baseline were observed at doses of 2.5-10 uM SAHA for 24-48 hours. Hut78 cells with lower baseline bcl-2 levels and higher baseline apoptosis showed 40-56% SAHA-induced apoptosis at 2.5-10 uM over 48 hours. SAHA treatment in both lines induced the cyclin-dependent kinase inhibitor, p21 (WAF1/CIP1) but did not alter the level of bcl-2 protein and there was no correlation between cell sensitivity to SAHA and basal expression levels of bcl-2. In 6 CTCL patients's PBLs treated with SAHA, increased apoptosis started at 0.5 uM and was at a maximum of 50% at both 5-10 uM for 48 hours. Sezary syndrome (SS) patients experiencing clinical responses to oral SAHA at 400-550 mg/day also showed a 50% reduction in the malignant T-cell clones in vivo as early as 4 weeks after starting treatment. SAHA is a new potential therapeutic agent for the treatment of CTCL that may work through inducing of T-cell apoptosis, independent of bcl-2.

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An FDA perspective on dermatologic drug development: The FDA Reviewer and Dermatology Advisory Committee Meetings

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FDA's Division of Dermatologic and Dental Drug Products at the Center for Drug Evaluation and Research reviews the majority of dermatologic drugs for safety and efficacy. Currently, the primary reviewers in the Division consist of dermatologists, dentists, pharmacologist/toxicologists, chemists, biostatisticians, and biopharmaceuticists. The FDA medical reviewer's role is to evaluate information submitted to New Drug Applications (NDAs) and conclusions from other disciplines for relevance. The dermatologist reviewer is crucial to evaluation of dermatologic drug products. FDA Advisory Committees make recommendations on issues related to FDA's regulatory responsibilities. The FDA reviewer is responsible for proposing issues, preparing and presenting the material related to the clinical safety and efficacy of the drug under discussion. The dermatologist reviewer distills the content of the NDA, literature, recommendations from the Advisory Committee and other information and recommends either approval or non-approval of dermatologic drug products. The issues that have been brought to Advisory committee are many and varied. A retrospective review of the topics brought to the FDA Dermatology Advisory Committee meetings over the past 20 years gives insight into the issues for which FDA dermatology medical reviewers evaluate and find solutions.

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Phenotypic reversion of melanoma *in vitro* suppresses metastasis *in vivo*

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Induced reversion of the melanoma malignant phenotype offers a theoretically novel approach to preventing growth and spread of biologically heterogeneous, therapeutically intractable tumors. Since lethality of most melanomas depends ultimately upon in vivo metastasis, we studied the effect on the latter of first inducing phenotypic reversion in vitro, using an endogenous inhibitor, Contact Inhibitory Factor (CIF), present in culture medium of a revertant line of melanoma cells. CIF was previously shown to restore normal in vitro growth control (density-, serum-and anchorage dependence) to mouse, hamster and human melanoma cells, and to also induce early G1 growth arrest, synthesis of pigment differentiation antigens, upregulation of class I MHC leading to enhanced destruction by cytotoxic T cells, suppression of angiogenesis at its source, and permanent regression of established melanomas in vivo. B16F10 cells were treated with or without CIF for 48 hours, the former inducing the characteristic morphologic changes accompanying reversion. Equal numbers of viable control and revertant cells were then employed in the chick embryo chorioallantoic membrane (CAM) assay, which measures the later stages of metastasis (extravasation and colony formation), with cells injected into 10 day old embryos, followed 1 week later by scoring of lung and liver metastases. In Experiment I, there was a significant difference in mean number of lung metastases (control 3.1 ± 3.0, N=11; CIF 0.5 ± 0.9, N=18; 0.003<P). In Experiment II, a similar result was seen (control 6.1 ± 2.5, N=8; CIF 2.2 ± 2.4, N=8; 0.006 <P). In both experiments there was a striking decrease in liver metastases with revertant cells. In conclusion, decreased ability to metastasize is an additional accompaniment of phenotypic reversion. Although the molecular changes mediating suppression of metastasis remain to be explored, induced reversion of the melanoma malignant phenotype is a valid target for future therapeutic investigation.

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Pimecrolimus and tacrolimus differentially affect genes related to skin inflammation in rats

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Contact hypersensitivity (CH) in laboratory animals has been used as model of a T cell mediated skin disease to profile pimecrolimus (PI) for the treatment of inflammatory dermatoses. CH, assessed by the increase in skin thickness 24 hrs after elicitation, was inhibited by 49% in rats after oral treatment with 25 mg/kg, administered 2 hrs before and at time of challenge with 2,4-dinitrofluorobenzene (DNFB). Surprisingly, no significant effect on skin thickening was observed with tacrolimus (TA) at the same oral dosage. In order to get a deeper insight into the different pharmacodynamic activities of the two compounds in that model, skin of animals challenged with DNFB and treated with PI or TA as well as skin from untreated control animals was analysed for gene expression using a microarray technology. The genes affected by DNFB ($p < 0.01$) were selected and their treatment-related changes studied. Many genes which have been induced or reduced in expression by the challenge (such as Gro alpha, MCP-1) were normalised in expression level by both, PI and TA. To understand the mechanism underlying the lacking anti-inflammatory activity of oral TA against CH, genes differentially affected by the two compounds were investigated. In contrast to TA, PI inhibited the expression of several genes related to inflammatory or fibrotic processes such as PF4, galectin 3, heme oxygenase, alpha 2 macroglobulin or versican. Thus, that set of genes which was affected by PI only may account for the clinically observed inhibition of the CH reaction. In conclusion, TA and PI exhibited a strong effect on the gene expression in the elicitation phase of DNFB-induced CH. The changes induced by TA were not related to a decreased CH reaction and the genomic data may indicate that the effectiveness of PI results from inhibition of genes involved in inflammatory processes that were not affected by TA.

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Intravenous low-dose ketamine inhibits itch-induced by histamine prick in humans

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The purpose of the study is to investigate the effect of intravenous low-dose ketamine on histamine-induced itch in human volunteers. Itch was induced by skin prick of 1% histamine on a volar aspect of the forearm in 22 healthy male volunteers (median age 34, range 26 to 51). The intensity of itch (Numerical Rating Scale, 0-10) was monitored every minute until it disappeared. The following parameters were assessed; the area of wheal and flare at 15 min of skin prick, the maximum itch intensity and its duartion. Either ketamine (2mg, K) or normal saline (5ml, NS) was injected intravenously 2 min prior to skin prick on different days with an interval of one week in a double-blind randomized order. A second generation antihistamine, azelastine (2mg/day for 3 days, A) was taken orally as a positive control afterwards. The effect of K on itch and skin response to histamine prick was compared with those of NS and A. The area of wheal (sqmm) was 39.7+/-15.8 (NS), 35.0+/-10.1 (K) and 28.4+/-9.9 (A). The area of flare (sqmm) was 551+/-317 (NS), 420+/-263 (K) and 118+/-96 (A). The maximum itch intensity (0-10) was 5.1+/-1.7 (NS), 4.1+/-1.6 (K) and 2.8+/-1.8 (A). The duration of itch sensation (min) was 9.6+/-3.8 (NS), 8.0+/-4.1 (K) and 6.9+/-4.2 (A). The number of the subjects who felt slightly sedated was 5 with K and 1 with NS. There were 7 other subjects who felt slight dizziness with K. These sensations disappeared within a few minutes. No serious side effects were seen throughout the experiment. Ketamine is an intravenous anesthetic with a NMDA receptor antagonizing effect, which makes the drug used as an analgesic. It is also reported to be effective for chronic persistent itch in patients with erythroderma. Though less potent than azelastine, ketamine was shown to decrease itch sensation induced by histamine skin prick in healthy volunteers. This result suggests the possible role of NMDA in the transmission of itch impulse within the central nervous system. Further trials with varying doses and in other itchy conditions may prove ketamine to be a possible antipruritic.

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Consensus from 4 studies—use of twice weekly fluticasone propionate can maintain remission in moderate to severe atopic dermatitis

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Four randomized placebo-controlled studies have consistently demonstrated that FP cream and ointment significantly reduce the risk of relapse of moderate to severe AD and prolong remission time when applied once daily, twice a week for up to 16 weeks. This regimen should be applied to all previously affected sites of AD, or to known sites of potential relapse, and used in combination with routine daily emollients. Crucial to this twice weekly treatment strategy is the prior stabilization of AD (i.e. cleared/almost clear, with a target lesion score of none or mild) by regular daily FP therapy for up to 4 weeks. In total, the 4 studies recruited 772 subjects, of whom, 429 (193 were children 3months-15yr) were randomized to twice weekly FP ointment or cream for up to 16 weeks. Addition of twice weekly FP to a routine daily emollient regime reduced the risk of relapse by 5 to 8-fold with FP cream and 2-fold with FP ointment. Median time to relapse of AD for FP (either formulation) was in excess of 16 weeks (study monitoring period) and for emollients plus placebo was 3-6 weeks. Twice weekly FP was well tolerated in all 4 studies throughout the long-term 16 week study period with no evidence of increased risk of skin atrophy or telangiectasia. By preventing recurrent relapses of AD and therefore reducing the need for acute bursts of daily topical steroid therapy, this twice weekly FP maintenance regime may have steroid sparing potential in patients with recurrent disease.

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Effects of epinastine hydrochloride on cutaneous nervous system

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Epinastine hydrochloride, an H1 antagonist commonly used in Japan, has clinically potent anti-pruritic effect for patients with severe itch such as atopic dermatitis. We have examined new aspects of pharmacological modes of action of this medicine except for antihistamine, especially effects of epinastine hydrochloride on cutaneous nervous system. The dorsal root ganglia of ICR mice were cultured in medium containing various concentrations of epinastine hydrochloride. The percentage of ganglia showing outgrowth of neurites to all cultured ganglia, the number of neurites per one ganglia and the total length of neurites from ganglia significantly decreased in a dose-dependent manner after addition of epinastine hydrochloride into the culture medium. The nerve fibers outgrowing from the dorsal sensory ganglia contained substance P as one of neurotransmitters, which were stained immunohistochemically. Epinastine hydrochloride significantly suppressed the release of substance P from nerve fibers in culture stimulated with capsaicin in a dose-dependent fashion. Furthermore, in vivo study revealed that the levels of both nerve growth factor and truncated form of the nerve growth factor receptor in the urine as well as in the saliva were significantly reduced in patients with atopic dermatitis treated with epinastine hydrochloride. These results suggest that epinastine hydrochloride possesses direct modes of action to cutaneous nervous system, which may in turn contribute to a marked relief of itch intensity in pruritic dermatoses such as atopic dermatitis.

1196

Imiquimod-induced $CD4^+$ T cell responses lead to long lasting erythema after regression of basal cell carcinomas and actinic keratoses

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Therapeutic options for basal cell carcinomas (BCC) and actinic keratoses (AK) including (kryo-)surgery and photodynamic therapy are still not fully satisfactory. Based on preliminary reports, we have initiated topical treatment of BCC and AK by 5% imiquimod cream (Aldara, 3M, Cergy Pontoise Cedex, France), an immune response modifier that induces the local release of various cytokines (IFN-α, TNF-α, IL- 1, 5, 6, 8, 10 and 12). To date, 10 patients (6 BCC, 4 AK, age range: 40-60yrs) have been treated once daily either 5 times a week (BCC), or 3 times a week (AK) for up to 6 weeks. All patients experienced local erythema which in 9 out of 10 (exception 1 AK) progressed to erosions at the site of imiquimod application. No systemic side effects, either with regard to clinical symptoms or laboratory value changes were observed. After 3-6 weeks, 100% of lesions presented with histologic clearence. Clinically, the process of tumor regression was accompanied by spontaneous scar-free healing of erosions leaving a persistant erythema for more than 4 weeks. Immunohistochemical analysis of biopsies of these post-treatment erythemas (n=3) identified a quite homogeneous picture. Located at the upper dermis, the inflammatory infiltrate mainly consisted of $CD3^+$ T-cells and a significant proportion of $CD68^+$mononuclear cells. There were only small numbers of $CD20^+$ B-cells. $CD56^+$ (NK-) cells were absent. With regard to T-cell subsets, activated ($CD45RO^+$) $CD4^+$ helper/inducer cells clearly dominated in all specimens over the CD8+ T-cell phenotype. Taken together, 5% imiquimod cream represents an efficient and largely safe BCC/AK therapy seemingly working via the induction of a local, tumor-specific $CD4^+$ T-helper response. However, post-treatment erythema is cosmetically irritating and, at least at this point, we cannot decide on the natural course of this long-lasting imiquimode effect.

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Etretinate improves sclerotic skin of systemic sclerosis

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There are few satisfactory effective treatments for skin sclerosis of systemic sclerosis (SSc). Recently we reported SSc patients treated with vitamin A derivative etretinate, which is used for psoriasis with the effect to normalize the epidermic cell turn-over time. The aim of this study is to evaluate the advantage of etretinate for skin sclerosis of SSc by the modified Rodnan total skin thickness score. We enrolled 32 cases whose sclerotic changes from first visits had been followed up. Twelve cases were treated with etretinate, including 5 cases together with other treatments that have or had reported they improved skin sclerosis of SSc, that is, systemic steroids, immunosuppressants, D-penicillamine, and/or bucillamine. Twenty cases were not treated with etretinate, including 13 cases together with other treatments. They were treated with 0.5mg/kg/day etretinate as the initial dose and it was tapered after skin sclerosis improved or when side effects appeared. It was considered as significant improvements that sustained score became less than 25% of the score when treatments had started. Sixty percent (3/5) of cases treated with both etretinate and other treatments and 85.7% (6/7) of cases treated with etretinate but not together with other treatments had definite significant improvements. Whereas, improvements did not occur in the cases who did not recieve both etretinate and other treatments. Only 7.7% (1/13) of cases treated with not etretinate but other treatments had improvements. In all the cases treated with etretinate, and in the cases with etretinate but not together with other treatments, the mean of the sustained score decreased significantly compared with that of the score when treatments was started (P•0.01). It was suggested that etretinate was a useful treatments for sclerotic skin of SSc.

1198

A novel method to assess efficacy of enzymatic wound debriding agents

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The in vitro determination of an enzymatic wound debriding agent's (EWD) efficacy may be accomplished by determining its ability to digest collagen, fibrin and elastin, the major components of wound eschar. Current methods allow demonstration of these activities in solution. However, solution phase tests do not provide a true measurement of an enzyme's capacity to digest eschar proteins in situ, where neither the enzyme nor the substrate may be present in solution. Analytical methods have been developed to directly test the in vitro efficacy of proteolytic enzymes formulated as ointments, the most common dosage form of commercial EWD. A hydroxyproline assay was performed to determine collagenolysis and labeled fibrin and elastin were used to assay digestion of these substrates. A papain/urea debriding ointment (Accuzyme®) was used as the model EWD to demonstrate the efficacy of the test system. Samples of Accuzyme, with and without urea, were also prepared and tested to demonstrate the method's capacity to allow the study of the contribution of cofactors in the formulation to specific proteolytic activities. Bovine type I collagen (native and heat-denatured) was used for the collagenolysis study; fibrinolysis, and elastolysis were determined using the labeled components, fibrin-FITC and elastin-Remazol. Closely mimicking in use conditions, the work showed the non-solution-based, direct-digestion method is a substantially improved method to study the in vitro efficacy of an enzymatic wound debriding agent.

1199

A phase II trial to evaluate the efficacy of topical 1% bexarotene (Targretin®) gel in patients with parapsoriasis (T_0 cutaneous T cell lymphoma)

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Parapsoriasis is a term for a distinct precursor stage ($T_0N_0M_0$) of cutaneous T-cell lymphoma (CTCL) and is defined by the presence of skin lesions clinically and/or histologically suggestive of, but not diagnostic of CTCL. Parapsoriasis has been reported to progress to unequivocal CTCL in up to 30% of cases. The identification of therapies with the capacity to induce complete responses in parapsoriasis will provide the rationale of developing a chemoprevention strategy for individuals at risk for progression to CTCL. To develop a rational therapeutic strategy for parapsoriasis, we have initiated an open label, phase II trial evaluating the efficacy and tolerability of topical 1% bexarotene (Targretin®) gel in patients with parapsoriasis. The secondary objective is to evaluate the antitumor host response in pre- and post- treatment skin biopsies. Bexarotene is a retinoid analogue (rexinoid) that binds preferentially to RXR retinoid receptors and has been approved for use in the treatment of CTCL. To date, 42 subjects were screened (21 parapsoriasis (T_0) / 20 CTCL (T_1) / 1 psoriasis) and 6 have been enrolled. Five have completed 16 weeks of topical treatment (2 weeks of daily application followed by 14 weeks of twice daily application) with a follow-up visit at week 20. 5/5 subjects had responses to therapy: 1 with a complete response and 4 with partial responses as determined by a composite assessment of index lesion disease severity. 2/5 responders demonstrated significant reduction in CD4:CD8 T-cells and increase in CD56 NK-cells from pre-(day 0) and post-treatment (week#16) skin biopsies. Cutaneous toxicity (erythema, scaling, pruritus) was noted in all subjects and resulted in dose reduction in 2/5 subjects. Topical 1% bexarotene (Targretin®) gel appears to be an active therapy for parapsoriasis.

1200

Non-invasive retrieval of message (mRNA) from stratum corneum of lesions of psoriasis via adhesive films

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Recently, via repeated application adhesive film, we learned that a significant amount of mRNA is retained in the stratum corneum (SC) and it reflects gene expression. To determine application to pathogenesis and treatment we studied retrieval of mRNA from the SC of patients with psoriasis. Adhesive films were repeatedly (x4) applied to an involved and uninvolved skin site on the extremities of 5 patients. RNA was extracted from cells adherent to the tapes (presumably SC) and quantified by non-competitive, quantitative RT-PCR using a fluorescence-based 5'nuclease assay on an ABI 7000. Each sample was reverse transcribed in triplicate and each cDNA amplified and quantified in duplicate; values were converted to RNA mass via standard curve from total RNA from human spleen. It is assumed that the relative amount of b-actin in human spleen RNA is identical to that in SC from the adhesive films. Using specific primers the mRNAs of TNF-a and IFN-g relative to b-actin were measured using real time PCR. RNA mass varied: for uninvolved sites, from non-detected to 138 ng and for involved sites from 1.2 to 44 ng. In 4 samples RNA was isolated from both uninvolved and involved sites. The levels of TNF-a and IFN-g in psoriatic lesions were normalized to adjacent non-lesional skin and fold increase calculated. A > four-fold difference, involved vs. uninvolved, is hypothesized as being significant. By this criterion, the 4 subjects with matched pairs of detectable RNA mass in involved and uninvolved SC had much higher levels of TNF-a levels in involved than in uninvolved sites, mean fold increase = 18.8, range = 8.9 to 42. Only 1 subject had > 4 fold increase in IFN-g in lesional SC. The SC contains message adequate to measure transcriptional changes in diseased skin and therewith becomes a non-invasive tool to assess pathogenesis and response to treatment. Epidermal Genetic Information Retrieval Adhesive Films (EGIRAF) provides a non-invasive technique to make such assessments.

1201

The safety of topical cyclosporin A conjugate (CGC1072) in the treatment of mild to moderate psoriasis

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Psoriasis can be effectively treated with the immunosuppressant Cyclosporin A (CsA), although this treatment is associated with nephrotoxicity and hypertension. The objective of this study was to determine the safety and preliminary efficacy of two dose formulations (0.4% and 4.0%) of a topical prodrug of CsA linked to a hepta-arginine (CGC1072)in patients with mild to moderate plaque psoriasis. Twenty-four subjects were randomized to receive one of the two strengths of CGC1072 and vehicle control. Treatments were self-applied in a blinded fashion to contra-lateral psoriatic plaques over 42 days. Psoriasis was evaluated by a psoriasis severity score (PSS), incorporating erythema, induration, and scale. CsA levels in the skin and blood were measured periodically. Twenty-one subjects completed the study. Blood CsA levels were not detected in any patient at any time point. There were no serious adverse events. Adverse events possibly related to the study drug occurred in 6 patients, transiently elevated creatinine and increase in WBC count in absence of localizing infection (high); increased WBC thought due to gingival infection (low); lower leg edema, tenderness and impetigo in treated leg followed by flaring of psoriasis (high); constipation and diarrhea, biopsied plaque on treated side slow to heal, and elevated non-fasting serum glucose and glucose in urine (low), transient systolic BP elevation (high) and itching (low). Reductions in the overall PSS and the erythema severity score were observed as early as day 14 when comparing the median difference from baseline of 4% CGC1072. By day 42, PSS as well as erythema and induration had improved. (p=0.06, 0.04, and 0.07, respectively). The median concentration of 4% CGC1072 in skin was comparable to that observed in patients using oral CsA (0.7 versus 0.8). This novel topical cyclosporin treatment, CGC1072 appears to be well tolerated, not absorbed systemically, and offers potential benefit in treating mild to moderate psoriatic patients without the associated systemic adverse effects.

1202

Safety and efficacy of a bilayered skin construct in full-thickness surgical wounds

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This was a prospective, randomized, controlled clinical trial assessing safety and efficacy of a living bilayered skin construct (BSC; Organogenesis, Canton, MA) in treating full-thickness surgical excision wounds. This is a report from a single site of a multicenter trial. We enrolled 31 patients needing excision of a non-melanoma skin cancer. They included 18 females and 13 males, with an age range of 44 to 84 years (average 67). Each was randomized to receive a single application of BSC or to heal by secondary intention. Primary endpoint was time to complete wound closure. Pain, cosmetic appearance and patient satisfaction were assessed using a 10cm visual analog scale (VAS). Wound quality was rated by the Vancouver Burn Scar Assessment Scale (VBSAS). Despite randomization, average wound size was larger for the BSC-treated group (mean area in cm^2: 9.45 for BSC and 5.79 for controls; P = 0.20). No statistical significance was seen between the two treatments: time to wound closure (mean number of weeks: 7.27 for BSC and 7.31 for controls; P = 0.86), cosmetic outcome (mean VAS reading: 8.16 cm for BSC and 8.17 cm for controls, P = 0.54), VBSAS rating (mean: 4.47 for BSC and 4.3 for controls; P = 0.69) patient satisfaction (mean VAS reading: 8.16 cm for BSC and 9.11 cm for controls, P=0.10), intensity of post-operative pain (mean: 0.46 for BSC and 0.85 for controls, P=0.44), post-operative pain resolution (mean number of weeks: 1.73 for BSC and 3.38 for controls, P = 0.072), and infection rate (0% for BSC and 25% for controls; P = 0.10). These findings indicate that BSC is safe in the treatment of acute excisional wounds and may facilitate management by decreasing post-operative pain.

1203

HO/01/2002, a new agent, dramatically improves wound closure and healing

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Heal-Or Ltd., has demonstrated the efficacy of a new therapeutic agent HO/01/2002 for accelerating the healing process of skin wounds. We completed preclinical studies using C57BL mice that were wounded and treated with various concentrations of HO/01/2002 versus vehicle treated control . Dose response as well as the age of the mice were tested. In order to demonstrate the changes in epidermal regeneration during stages of wound healing, we performed morphological, histological and immunohistochemical analysis. We examined wound biopsies at days 1-7 following wounding, utilizing antibodies to keratin 14 (basal layer), keratin 1 (spinous layer), Filaggrin (granular layer) and keratin 6 (wound healing & migration). From H&E stained sections, it is clear that in comparison to vehicle treated controls, HO/01/2002 treated wounds are smaller and spatial differentiation of the epidermis is well organized. The wound gap and the newly forming epidermis were analyzed by keratin 14 staining. Within 7 days, the epidermal gap of treated wounds is at least 2 folds smaller than control. Moreover, in control wounds within 4 days migrating cells of the newly formed epidermis at the wound gap preferentially expressed keratin 14 while keratin 6 expression was restricted to the wound edges. Interestingly, HO/01/2002 induced keratin 6 expression in the migrating keratinocytes of the newly formed epidermis underlying the wound gap. In treated wounds spatial differentiation was reconstructed in the newly formed epidermis as observed by keratin 1 staining. In contrast, no change in distribution or expression of filaggrin was observed in control versus HO/01/2002 treated wounds. Overall, the results demonstrate that The agent is a wound healing accelerator that has remarkable effect on wound closure by enhancing reconstruction of the epidermis and affecting migration and differentiation properties of keratinocytes at the wound gap. In order to demonstrate the benefit of HO/01/2002 on diabetic foot ulcers, we are currently performing experiments on diabetic wound healing impaired mice.

1204

Infliximab therapy in scleroderma patients with complicated Raynaud syndrome: an open label study

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To evaluate effectiveness of infliximab in patients with scleroderma.The following parameters were analysed for efficacy:joint count,visual analogue scale (VAS) for Raynaud phenomenon,Rodnan scale of skin involvement and short-form SF36 for quality of life. Case 1: A 25-years-old woman,diagnosed of Scleroderma 7 years old previously,scl 70+.Hospital admissions were requiered in 2 consecutive years for Raynaud therapy with prostaglandins.She had seronegative polyarthritis with advanced deformities unresponsive to disease-modyfying drugs,included methotrexate.After 3 infusions of infliximab at 5mg/kg she reported mild improvement.Case 2: A 43-years old woman with CREST syndrome diagnosed 5 years previously,had been treated as inpatient for severe Raynaud in 2 consecutive years.She has received 6 infusions of Infliximab 5 mg/Kg with subjective improvement.Case 3: A 51-years old woman was diagnosed of diffuse scleroderma with visceral involvement,pericarditis and intersticial pneumonitis.She was treated with cyclophosphamide for 2 years.After single Infliximab infusion developed heart failure. A echocardiograme demostrated restrictive cardyomiophaty. No further infusions were administered. Only patients 1 and 2 were evaluated for eficaccy.VAS for RAynaud reduced 40% in patient 2.None of them have required new admissions for Raynaud complications.Quality of life by SF36 items experienced improvement:physical function 25%,physical role 12,5%,body pain 36%,vitality 10%,social function 37,5%,mental health 20%.Joint count,Rodnan scale and patients and physician global evaluation didn't show significative differences. No new admissions were necessary for Raynaud complications.No improvement in joint count was obtained,but SF36 showed improvement in the majority of items.We think that better results would be obtained in less evolutioned disease.Subclinical cardiomyophaty should be rouled out before infliximab treatment.

1205

Phenol and trichloroacetic acid peeling is a new tool as non-invasive therapy to the aged patients with skin cancer

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Recetly the number of the aged patients with skin cancer has been increased in Japan. The problem is that many aged patients refuse the operation. For such patients, non-invasive therapies might be useful such as topical therapy, photodynamic therapy, cryosurgery and laser therapy. In this study we focused the chemical peeling using phenol and trichloroacetic acid(TCA). First, we examined the histological changes of normal human skin treated with 40-60%TCA, phenol and liquid nitrogen. On day 2 or 3, phenol had more severe degenerative changes than TCA. Interestingly, phenol penetrated deeply into skin and induced Tunel-positive degenerations in endthelial cell of dermis. However, TCA induced the degenerations from epidermis to dermis in the dose-dependent way without endothelial changes. On day 7, reepithelization was almost completed in cases of TCA and phenol, but inflammmatory cells were present in both peeling. In contrast, liquid nitrogen had less effects compared with TCA and phenol. From January 2001 to May 2002, 268 aged patients with akin tumor visited our Dermatology clinic. Out of them, 37 patients refused the operation or had the difficulty in the surgery. After informed consent, chemical peeling was performed to 3 seborrheic keratosis(SK), 14 actinic keratosis(AK),10 basal cell carcinoma(BCC), one trichoepithelioma, 5 Bowen's disease, 3 genital Paget's disease and one squamous cell carcimoma(SCC). More than 1 year follow-up after peeling was done in 15 cases. Thirteen cases showed complete response(CR) which was evaluated clinically and histlogicaly. Two cases without CR were cases with genital Paget's disease. The mean peeling time were 3.7 in AK, 6.5 in BCC, 6 in trichoepithilioma, 8 in Bowen's disease and 6 in SCC. We concluded that chemical peeling was useful for the aged patients with skin cancer.

1206

Altered gene expression in normal human epidermal keratinocytes by retinoic acid isomers and their 4-oxo-metabolites: 4-oxo-13cis RA is the most potent derivative

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Vitamin A and its natural and synthetic derivatives (retinoids) exert profound effects on fundamental processes such as cell proliferation and differentiation. In this experiment RA isomers and their 4-oxo metabolites were incubated with cultured NHEKs for 24 hours and analysis of differentially expressed genes was performed using DNA-Chip-technology. We examined 4400 sequence-validated cDNAs and ESTs arrayed on DermArray GeneFilters microarrays. Incubation of cells with 10-5M of the respective retinoic acid isomer for 24 hours revealed the upregulation of keratin 13 and keratin 7 and metallothionein and downregulation of keratin 10 by 13cis-, all-trans- and 9cis-RA. Expression of TIMP1 and collagen XVIII, is downregulated by 13cis and all-trans-RA but upregulated by 9-cis-RA. Expression of MMP7 is downregulated by 13cis and all-trans-RA but expression is not mediated by 9cis-RA. These data reveal that 13cis- and all-trans-RA have a similar effect on the expression of genes related to extracellular matrix compounds and metabolism, whereas 9-cis seems to have the opposite effect. Incubation of cells with 10-5M of the 4-oxo-metabolites for 24 hours revealed similar expression profiles compared to their parent compounds. 4-oxo-13cis- and 4-oxo-all-trans-RA also had a similar effect on the expression of genes related to extracellular matrix compounds and metabolism, whereas 4-oxo-9-cis RA seemed to have the opposite effect. Concerning the number of differentially regulated genes, 4-oxo-13cis RA seems to be the most potent compound. This substance was able to up- or down-regulate more than twice as many genes as the other RA isomers or 4-oxo-metabolites. Interestingly, these 4-oxo metabolites showed a comparable but not completely similar gene expression profile in comparison with their respective parent compound.

1207

High clinical response rate with multi-modality immunomodulatory therapy for Sezary syndrome

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Multi-modality immunomodulatory therapy for the treatment of Sezary syndrome (SS) involves the use of biologic response modifiers in combination to enhance cell-mediated immunity and promote tumor regression. Patients with SS exhibit profound defects in cell-mediated immunity characterized by deficiencies in cellular cytotoxicity and Th1 cytokine production. In an attempt to enhance anti-tumor immunity and correct the associated immunologic defects, we have utilized this treatment approach for the management of patients with erythroderma and peripheral blood involvement (>10% Sezary cells). Since January 2000, 27 patients with SS were treated with multi-modality immunomodulatory therapy at the Hospital of the University of Pennsylvania. Therapy consisted of extracorporeal photophoresis (ECP) in combination with two or more systemic biologic response modifiers: IFN-alfa, IFN-gamma, bexarotene, isotretinoin, sargramostim, and/or PUVA. All patients received >6 cycles of ECP, with the exception of two. Among the clinical responders (93% of patients), 82% had a $\geq$50% reduction in circulating Sezary cells. Thirty percent of patients achieved a complete clinical response characterized by no evidence of cutaneous disease and a Sezary count $\leq$5%. Sixty-three percent of patients achieved a partial response defined by at least a 50% reduction in cutaneous and blood disease. Only two patients exhibited no response to therapy. Based on our experience of a 93% response rate, multimodality immunomodulatory therapy has proven to be a dramatically effective treatment for the management of CTCL patients with advanced disease. The durability of response and impact on overall survival remain to be determined, however, this approach offers an appealing alternative to treatments with higher associated morbidities.

1208

NFκB is a possible target to inhibit ultraviolet irradiation induced hypermelanosis

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Vitamin D3 is one of the commonly used dermatological therapeutics, which markedly improves psoriatic lesions. The use of vitamin D3 with ultraviolet irradiation has been reported to be more effective than vitamin D3 or UV monotherapy on psoriasis. The use of vitamin D3, however, sometimes causes stronger pigmentation. In order to clarify the mechanism of pigmentation of topical use of vitamin D3 combined with UVB irradiation, we utilized 3-demensional epidermal models containing melanocytes. UVB irradiation upregulated the number of melanocytes, which was not significantly affected by the addition of vitamin D3. Electron microscopic observation revealed that the number of stage 4 melanosomes per unit area of melanocyte cytosol was significantly higher in the melanocytes incubated with vitamin D3. We have tested the effect of topical application of a novel NF κ B activation inhibitor, IMD-0354, on the hypermelanosis model induced by UV irradiation. IMD-0354 (M.W. 384) was designed starting from a lead compound discovered by virtual screening based on a modeled protein structure of IKK β and measurement of inhibitory effect on NF κ B. IMD-0354 inhibits NF κ B activation stimulated by TNF α at the concentration of 0.05 μM and also releases of various inflammatory mediators. It was confirmed that the compound inhibited neither kinases nor receptor bindings related to immune responses. As a result of the test, the addition of this compound significantly inhibited the UVB induced upregulation of melanocyte numbers. The above results suggest that vitamin D3 promotes melanocyte differentiation rather than proliferation, and that stronger pigmentation after UV therapy combined with vitamin D3 may be the cause of differentiation of melanocytes rather than proliferation. Inhibition of NF κ B activation is a possible target to prevent hypermelanosis after UV irradiation, and the topical application of IMD-0354 may be one of the possible means to decrease post-UV hypermelanosis.

1209

The effect of a topical oxygen emulsion on collagen deposition and angiogenesis in second-degree burn wound repair

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Oxygen is a required substance for wound healing. A pig model of second-degree burn wound was used to evaluate the effect of a newly developed topical aqueous oxygen emulsion (AOE) on collagen synthesis and angiogensis during wound repair. Burn wounds were treated with vehicle control, AOE (which contains super-saturated oxygen) or air exposure (no treatment). Wound tissue samples were collected at days 1, 4, 7, 10, 14 and 21 after wounding. Semi-quantitative RT-PCR (Reverse Transcription and Polymerase Chain Reaction) was used to examine the pattern of changes at mRNA levels for collagen I, collagen III and vascular endothelial growth factor (VEGF). Our preliminary data from 2 pigs showed: 1) Higher expression of collagen I, collagen III and VEGF m-RNA in the AOE treatment group. 2) Collagen III m-RNA expression increased earlier after wounding with a peak of dramatic increase at day 10 and day 14. 3) Collagen I expression level increased later than that of collagen III with the highest peak at day 14 and second higher level at day 21. 4) There was no significant difference between AOE and control groups at day 21. The data suggests that sustained high level of oxygen release by AOE may promote the process of wound repair through the mechanism of increased expression of collagens type I and type III and VEGF, which is critical in granulation tissue formation during wound healing.

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Combination chemotherapy for the treatment of ultraviolet light B-induced skin tumors using Efudex and Celecoxib

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Despite the alarming number of non-melanoma skin cancer (NMSC) cases diagnosed every year, standard chemotherapeutic agents used for the treatment of these lesions and precursor lesions are not completely effective. Increasing evidence suggests that repeated inflammatory sunburn reactions, which include the induction of cyclooxygenase-2 (COX-2) and the subsequent production of prostaglandins, play a role in skin cancer development. Although data generated in our laboratory as well as others has shown COX-2 inhibition to be an effective means of preventing skin cancer development in mice, these studies suggested that COX-2 inhibitors alone are not effective as chemotherapeutic agents, or for inducing the regression of established tumors. Limiting the proper use of and hence the effectiveness of current therapies for skin cancer are the severe inflammatory side-effects that result from their use. Due to these side-effects and the increasing data suggesting greater efficacy with combination therapies in a variety of cancer types, we hypothesized that a combination of 5-fluorouracil (5-FU; Efudex, ICN Pharmaceuticals, Inc.) in conjunction with the COX-2 inhibitor and anti-inflammatory drug Celecoxib (Celebrex, G.D. Searle & Co.) would act synergistically to regress tumors in a murine model of ultraviolet light B (UVB) induced carcinogenesis. In fact, we found that depending on the dose used, a combination of topical 5-FU (1% or 2.5% Efudex) in combination with Celecoxib (1 or 2 mg) is up to 35% more effective in reducing the number of UVB-induced skin tumors than 5-FU treatment alone. In addition, animals treated with the combination therapy had a lower rate of recurrence compared to 5-FU treatments alone three weeks after the cessation of treatments. This data provides hope that more effective chemotherapy regimens can be developed to treat the millions of precancerous and cancerous skin lesions that arise every year, which could ultimately lead to a significant reduction in costs and cosmetic defects (scarring) associated with surgical interventions.

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Preventing the effector phase of allergic contact dermatitis with a metabolic inhibitor of CLA synthesis

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E- and P-selectin on post-capillary venules in inflamed skin play a critical role in the migration of effector T-cells. Cutaneous lymphocyte-associated antigen (CLA), a specialized glycoform of P-selectin glycoprotein ligand-1 (PSGL-1), and a fucosylated PSGL-1 equivalent are the principal selectin ligands on human and murine skin-homing T-cells, respectively, and are required for T-cell entry into inflamed skin. We have previously shown that a fluorinated analog of N-acetylglucosamine, 4-F-GlcNAc, inhibits CLA expression and function. To investigate the in vivo efficacy of 4-F-GlcNAc on a murine model of cutaneous inflammation, we performed delayed-type hypersensitivity (DTH) experiments in mice treated with 4-F-GlcNAc (i.p.; 50-250mg/kg q.d. X 6). In addition, to analyze the effects of 4-F-GlcNAc on antigen-recognition and/or effector phases of DTH, we examined the migration of Langerhans cells (LC) from sensitized skin to draining lymph nodes (LN), and performed flow chamber studies on selectin ligand activities of Th1 cells and Stamper-Woodruff assays on LN high endothelial venule (HEV) L-selectin ligand activities. We found that elicitation of DTH was prevented with 50mg/kg 4-F-GlcNAc ($p<0.01$, when compared with controls), while no differences in HEV L-selectin ligand activities were observed. Furthermore, we observed no difference in the capacity of antigen-presenting LC to migrate to draining LN. However, flow chamber studies revealed that 4-F-GlcNAc (50μM) completely inhibited the capacity of Th1 cells to bind E- and P-selectins ($p<0.001$; when compared with controls). These data indicate that 4-F-GlcNAc prevents DTH by inhibiting selectin ligand activity of Th1 cells. Moreover, 4-F-GlcNAc does not appear to affect the afferent phase of DTH, as there was no effect on LC migration to draining LN and on the capacity of HEV to support lymphocyte entry into draining LN. The profound and specific effect of 4-F-GlcNAc on dermal lymphocytotropism suggests that 4-F-GlcNAc could be beneficial in long-term treatment of chronic cutaneous inflammation.

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Lidocaine skin penetration profile using novel foam formulations

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Topical lidocaine has been widely used as a local anesthetic, usually delivered in the form of cream or ointments. Foam formulations have emerged as a superior vehicle because they leave little sticky or greasy residue on skin. This study investigated the effects of novel foam formulations on in vitro skin permeation of lidocaine. Two novel foam formulations containing 1% and 5% lidocaine (base) were tested for percutaneous penetration on human excised skin (dermatomed to 250 um).The penetration was monitored by collecting receptor fluid samples every 15 minutes in the first hour and every 30 minutes subsequently.Lidocaine content in the receptor fluid, surface wash, epidermis and dermis was measured by scintillation counting. A commercial product containing Lidocaine base, ELA-max 4% cream, was also studied for comparison. The penetration rate of radiolabeled lidocaine from the 5% foam formulation was higher than that from the 1% foam formulation. Application of 5% lidocaine in the foam formulation resulted in a similar penetration profile as the commercial product (4% lidocaine cream). The presence of lidocaine in the receptor fluid was observed starting at 30 minutes post-application of both 5% lidocaine foam and 4% lidocaine cream, whereas it was observed later for the 1% foam. The 5% lidocaine foam formulation has a penetration profile similar to the commercially available 4% lidocaine cream product and, therefore, is likely to show the same onset of action. As the foam formulation provides an elegant mode of application, it may be an attractive product for delivery of lidocaine with an efficiency similar to currently listed products.

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Skin penetration profile of clobetasol propionate from foam, cream and ointment

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Clobetasol propionate (CP) has been used as a superpotent steroid (Class I) for the treatment of various skin inflammatory diseases. As formulations have been known to influence the delivery of the drug, it is of interest to study the effect of formulations on in vitro skin permeation profile of CP. Method: A foam formulation (OLUXTM), an ointment (TemovateTM Ointment) and a cream (TemovateTM E Cream), each containing 0.05% CP, were tested for percutaneous penetration on human excised skin. A dose of 5-10 mg/cm2 of each formulation, spiked with radiolabeled 3H-clobetasol propionate, was applied for 48 hours using In-line cells. Penetration was monitored by collecting receptor fluid samples every 4 hours. At the end of application time, the skin surface was washed, wiped and stripped, then the epidermal layer was split from the dermis. Drug content in the receptor fluid, surface wash, epidermis and dermis was measured by scintillation counting. Results: The foam and cream were shown to reach the maximum delivery rate of CP at 4 to 8 hours post-application, faster than the ointment (12-20 hours). However, the foam had a maximum rate 4 times higher than that of the cream. The ointment maintained a higher rate much longer than the other forms, showing a flux at 48 h still at 70% of the maximum. At the 12 h time point, foam delivered cumulatively the most drug into the receptor fluid, 400% more than cream (p value) and 15% more than ointment. On the other hand, at 48 h, the ointment delivered twice as much CP as the foam and 5 times more than the cream. The cream resulted the lowest penetration rate and amount among the three dosage forms. Conclusions: The foam formulation delivered the most CP within the first 12 hour period, and subsequently, the penetration rate went down quickly, with minimal residual steroid remaining in the skin. The foam may be a better alternative to the ointment and cream both for short and long-term therapy with maximum efficacy and minimum adverse reactions.

1214

Chemosensitization of melanoma cells by proapoptotic genes

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One of the obstacles in metastatic melanoma treatment is its resistance to conventional chemotherapy. The p53 tumor suppressor has been shown to be mutated in 50% of human cancers, but only approximately 15% in melanoma biopsies, suggesting that other factors may mediate melanoma chemoresistance. To investigate the role of cell-death inducing proteins such as Apaf-1 (Apoptotic Protease Activating Factor-1) and PUMA (p53-Upregulated Modulator of Apoptosis) in their involvements in melanoma chemosensitivity, we overexpressed proapoptotic genes PUMA and Apaf-1, a downstream effector in the pathway, and assessed effectiveness in sensitizing melanoma cells to drug treatment. We demonstrated that the introduction of PUMA was able to sensitize melanoma cells by decreasing cell survival to 64% or nearly 34% after camptothecin treatment when compared to the baseline GFP-transfected control cells, where survival was 99% and 70%, respectively ($P<0.0001$; $P<0.0001$). We further showed that PUMA overexpression induces apoptosis and not necrosis by DNA-laddering. In addition, we demonstarted that overexpression of Apaf-1 in melanoma cells was able to decrease cell survival to 73% ($P<0.05$) alone and nearly 39% after camptothecin treatment ($P<0.005$) when compared to the baseline GFP-transfected control cells. The combination of PUMA/Apaf-1 and camptothecin has an additive effect on cell survival. This project will continue to explore more effective treatment regimes, at the same time to provide new insights in the mechanisms of chemoresistance in malignant melanoma.

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***Arctii fructus* is a prominent dietary source of linoleic acid for reversing epidermal hyperproliferatin of guinea pigs**

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Dietary deficiency of linoleic acid [LA; 18:2(n-6)], the major polyunsaturated fatty acid in human epidermis, induces epidermal hyperproliferation. The recognition that LA is important to maintain normal skin has been associated with metabolism of LA in the epidermis. Because of the inability of normal epidermis to desaturate LA to γ-linolenic acid, it is metabolized by epidermal 15-lipoxygenase to mainly 13-hydroxyoctadecadienoic acid (13-HODE), which exerts antiproliferative biopotency. Since safflower oil has been widely recognized as the major dietary source of LA and Arctii Fructus (Arctium lappa L.) is recently reported to contain high level of LA, we compared the antiproliferative effects of safflower oil and Arctii fructus in this study. Essential fatty acid deficiency (EFAD)-induced epidermal hyeprproliferation was employed in guinea pigs by hydrogenated coconut oil (HCO) diet for 8 wk. During 2 wk, EFA deficient guinea pigs were fed safflower oil (group HS), water extract of Arctii Fructus (group AW) or organic extract of Arctii Fructus (group AO) containing diets. Since water extract is commonly used in oriental medicine, group AW was included in this study. Normal control group was fed safflower oil (group SO) containing diet and EFA deficient group was fed HCO containing diet for 10 wk. By determining the thymidine incorporation, our data revealed that epidermal hyperproliferation was reversed in groups AO (55.9% of group HCO) and HS (74.1% of group HCO). Epidermal hyperproliferation was not reversed in group AW. The accumulations of LA into phospholipids and ceramides, and of 13-HODE, the potent antiproliferative metabolite of LA in the epidermis of group AO were greater than of group HS. Together, our data demonstrate that Arctii Fructus exerts higher antiproliferative biopotency than safflower oil with higher accumulation of LA and 13-HODE in the epidermis of guinea pigs.

1216

Anti-CD20 monoclonal antibody therapy for primary cutaneous B cell lymphomas

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CD20 is a nonglycosylated phosphoprotein present on mature B cells and >90% of B-cell lymphomas. It is critical in proliferation and differentiation, acting possibly via a Ca^{2+} channel. Primary cutaneous B-cell lymphomas (PCBLs) constitute a heterogeneous group of rare B-cell derived lymphoproliferative disorders with variable survival rates and high incidence of local recurrence with surgery and/or local radiotherapy. The chimeric anti-CD20 monoclonal antibody rituximab induces B-cell complement-mediated cytotoxicity in vitro, and transient B-cell depletion in vivo. We report complete responses to rituximab in 2 cases of low and intermediate aggressive clinical behavior PCBCLs. Staging was performed by total body CT, laboratory studies, and bone marrow biopsy. After informed consent was obtained, both patients received 4 weekly infusions of 375mg/m^2 body surface intravenous rituximab. Patient 1 presented with 0.5-2.0 cm red-brown nodules on his face. Skin biopsy showed a dermal infiltrate of small atypical CD20+ lymphocytes, compatible with folliculocentric lymphoma. Despite electron beam radiation therapy (EBRT), relapse occurred 7 months later. Rituximab was then initiated. Patient 2 presented with 3x2 cm violaceous nodules on his left thigh. Skin biopsy showed large CD20+ cells in the dermis and hypodermis, consistent with large B-cell lymphoma. Because treatment with PUVA and EBRT did not alter progression, rituximab was given. There were no serious adverse drug reactions, and complete response was achieved in both patients. There was no relapse on evaluations at 10 and 5 months, respectively. Few cases of primary CBLs treated with with rituximab have been reported, with variable responses. Rituximab is a high-affinity monoclonal antibody with a murine variable anti-CD20 region and a human IgG1 constant region, the latter accounting for its low immunogenicity. Our observations support the treatment of relapsing PCBCLs with systemic rituximab, as an effective and well-tolerated therapy.

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Open trial of topical tacalcitol [1α24$(OH)_2D_3$] and solar irradiation for Japanese patients with vitiligo vulgaris: up-regulation of c-Kit mRNA by cultured melanocytes and nerve growth factor production by cultured human keratinocyte cell line

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Vitiligo vulgaris is a common skin disease, however some cases show poor clinical responses to topical steroid ointment or PUVA therapy. Such regimens are generally avoided in the treatment of facial lesions or in pediatric cases because of the undesirable side effects. Dysregulation of c-Kit signaling has been thought to be one candidate in the pathogenesis of vitiligo vulgaris. To confirm the excellent response to combination therapy with topical vitamin D_3 ointment and solar irradiation for vitiligo achieved in the initial patients, we conducted an open trial on other patients, most of whom had poor clinical responses to the prior therapies. Fifteen patients (9 men and 6 women) with vitiligo vulgaris were enrolled in this study. Each patient was instructed to take sunbath for 30 minutes within 1 hour after topical application of the tacalcitol [1α24$(OH)_2D_3$] ointment or cream to the skin lesions every day. Six of 15 patients showed fair and excellent clinical response to the combination therapy (more than 30% clearance of the vitiligo). The clinical effect was more apparent in patients with a history of less than 5 years of vitiligo (4 of 6 cases) in contrast to those with a history of more than 5 years (2 of 9 cases). *In vitro* experiments revealed that tacalcitol upregulated the expression of c-Kit mRNA by melanocytes irradiated with linear polarized infrared, UVA or short period solar irradiation. In addition, the tacalcitol [1α24$(OH)_2D_3$] upregulates one of the melanocyte growth factor, NGF mRNA and protein by cultured human keratinocytes. These results suggest that combination therapy with topical vitamin D_3 ointment and solar irradiation can be used as an alternate therapy for vitiligo vulgaris.

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Down-regulation of cyclin A in squamous cell carcinoma line, SCC13 by PPAR-selective ligand

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily. Recent evidence has suggested that PPARs regulate cell growth and differentiation in many cell types including malignant neoplasm cells. In the present study, we investigated: 1) the expression of PPARs in squamous cell carcinoma line, SCC13; 2) the growth inhibitory effect of GW362433, a PPAR α/β selective ligand, on SCC13 cells; and 3) the molecular mechanism of GW362433 induced cell-cycle arrest. Expression of PPAR mRNA was detected in several squamous cell carcinoma lines using Northern blot analysis. A cytostatic effect of GW362433 was found in several squamous cell carcinoma cell lines, including SCC13, SQ/CCY1, HaCaT, and HPV18-transformed NHEK cells. This growth inhibition was dosage-dependent. Flow cytometric analysis demonstrated that GW362433 induced a G0/G1 arrest in SCC13 cells. The mRNA and protein expression levels of cyclin A, which expression correlates with the transition of the G1 to S phase of the cell cycle, were analyzed by Northern and Western blot analysis, respectively. Cell cycle arrest in SCC13 induced by GW362433 was associated with a dramatic decrease in the level of cyclin A. Our data indicate that squamous cell carcinoma express functional PPARs and that PPAR activation by GW362433 induces growth inhibition associated with G1 cell cycle arrest in SCC13. Our results also indicate that the reduction in cyclin A expression is an important indicator in the inhibition of cell growth by GW362433. The growth inhibitory effects exhibited by GW362433 suggest that certain PPAR ligands could be useful in the treatment of human squamous cell carcinomas and other hyperproliferative skin diseases such as psoriasis.

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Dose-escalating study of intratumoral administration of adenovirus-interferon-γ (TG1042) in primary cutaneous T and B cell lymphomas

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Primary cutaneous lymphomas (CL) have been successfully treated with interferons (IFNs) counterbalancing the T_h2-skewing state. We undertook a phase I, open-label, dose-escalating trial of repeated, intratumoral administration of TG1042 in patients with advanced primary cutaneous T cell lymphomas (CTCL) and multilesional cutaneous B cell lymphomas (CBCL). TG1042 represents non-replicating recombinant adenoviral vector containing human IFN-γ cDNA insert. Nine patients (7 CTCL, 2 CBCL) were enrolled in 3 successive cohorts at the following TG1042 doses: $3\neq10^9$ total particles (tp), $3\neq10^{10}$ tp and $3\neq10^{11}$ tp. The injected lesion was biopsied at baseline and after 3 injections, and assessed for changes in lesion's morphology, expression of CD1a, 3, 4, 8, 20, 21, 56, 79a, TIA-1, coxsackie-adenovirus receptor (CAR) and HLA class I. In addition, the lesions were evaluated for transgene-derived IFN-γ expression as well as for IFN-γ, IL-2, IL-13, IL-10 production by quantitative PCR. Local clinical response has been observed in 6 out of 9 evaluable patients. Injection site reaction was the most commonly observed adverse event. Following TG1042 injections, histology demonstrated pronounced changes in infiltrate pattern differing from initial lymphoma finding, with signs of vasculitis and increase in eosinophil and neutrophil numbers. Immunohistochemistry revealed increase in CD8 and TIA-1 immunoreactivity. All injected lesions demonstrated clear up-regulation of CAR expression. Quantitative PCR showed decrease in CD4/CD8 ratio in 6/8 patients. Transgene-derived IFN-γ mRNA could be detected in injected lesions, implying successful IFN-γ gene transfer. Our results reveal for the first time the in situ immunological changes following the administration of adenoviral vector expressing IFN-γ in primary CTCL and CBCL, offering new insight and information of importance to the design of new gene delivery-based therapeutics in cancer.

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Treatment of atopic dermatitis with the xenon chloride excimer laser

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We demonstrated earlier that the 308 nm xenon chloride (XeCl) ultraviolet B (UVB) laser is more effective in treating psoriasis and inducing T cell apoptosis than narrow-band UVB. We also found that the excimer laser induced repigmentation in localized vitiligo. The aim of this study was to investigate the effectiveness of the XeCl laser phototherapy in atopic dermatitis. Five patients with flexural atopic eczema - less than 20 % body area involvement - were included in the study. We used the eczema area severity index (EASI) to characterize the severity of the disease. Before the laser treatment the scores were between 5.9-11 (mean 5.94). For the 308 nm laser therapy we used the XTRAC laser (Photomedex). The energy of each light impulse was 5.5 mJ/cm2, the diameter of the light spot was 3 cm. The initial dose was 50 mJ/cm2 less than the minimal erythema dose (MED). The dose was increased by 50 mJ/cm2 each week. The study was conducted in a one month period. The mean cumulative dose was 2 J/cm2. At the end of the treatment period the EASI scores were between 0-4.6 (mean 2.0) significantly lower compared to the initial values. These results suggest that the XeCl excimer laser is effective in treating localized atopic dermatitis. The 308 nm XeCl laser, this super narrow band UVB light source might well be the choice of treatment in different T cell mediated diseases.

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Role of mitochondria in the therapeutic action of anthralin

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Anthralin (dithranol) is an established safe and effective topical treatment for psoriasis. Anthralin induces biochemical, structural and functional changes to mitochondria and respiratory deficient mutant yeast cells (petite), which lack mitochondria DNA and cytochrome oxidase, are significantly more resistant to the cell killing effects of anthralin than respiratory competent yeast cells (grande). In this study, we examined the effect of anthralin on cell growth, mitochondrial membrane potential (MMP) and mitochondrial morphology in a human 143B ρ^0 osteosarcoma cell line lacking mitochondrial DNA, cytochrome oxidase activity and respiratory competent mitochondria, the isogenic cell parental cell line (143.TK$^-$) and normal human keratinocytes. 143B ρ^0 cells were significantly more resistant to the killing/growth inhibitory effects of anthralin following 72h treatment, compared to the parental cell line (median EC_{50} 87μM and 0.087μM respectively, P,0.05, n=5), as assessed using a Sulforhodamine B assay. Cells were loaded with a cationic dye JC1 and MMP assessed by laser scanning confocal microscopy. Anthralin reduced MMP in the parental cells in a time (30min-24h) and concentration (0.1-10μM) dependent manner. 143B ρ^0 cells maintain a lower MMP through glycolytic metabolism but this was unaffected by treatment with anthralin, up to 10μM for 24h. In cultured human keratinocytes, anthralin (1-5μM) reduced MMP as early as 30mins that persisted up to 24h. In the parental cells and human keratinocytes exposure to anthralin resulted in morphological changes to mitochondria from filamentous to swollen and more rounded structures which aggregated around the nucleus. This data indicates that i) respiratory competent mitochondria dramatically increase the sensitivity of human cells to the growth inhibitory/cell killing effects of anthralin and ii) the reduction of MMP induced by anthralin is dependent on the ability of cells to perform oxidative respiration and also occures in norman human keratinocytes at therapeutically relevant concentrations.

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Genistein enhances the cisplatin-induced inhibition of cell growth and apoptosis in human malignant melanoma cells

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Genistein, a naturally occurring isoflavone found chiefly in soybeans, has been reported to be a potent antitumor agent. Genistein is presumed to exert multiple effects related to the inhibition of cancer growth. Metastatic melanoma is a chemotherapy-refractory neoplasm. The present study was designed to explore the possible activity of genistein to inhibit the aberrant proliferation and to induce apoptosis of human malignant melanoma cells in cooperation with cisplatin treatment. Five human melanoma cell lines were utilized for these experiments. Genistein at physiologic concentrations (20 microM) did not induce apoptosis by itself but did enhance cisplatin-induced apoptosis in all 5 human melanoma cell lines tested. The enhanced susceptibility among the cell lines was diverse. The maximum effect of the combined treatment on the induction of apoptosis in MeWo melanoma cells was 3.8 times higher than untreated controls. The maximum effect on the enhancement of apoptosis in A101D cells was 1.4 times more than that of cells treated with cisplatin alone. Changes in the expression of two anti-apoptotic proteins, bcl-2 and bcl-xL, and one pro-apoptotic protein, Apaf-1, were examined. Genistein alone did not alter bcl-2 expression, but decreased bcl-xL expression and slightly increased Apaf-1. The combined treatment with genistein and cisplatin significantly reduced bcl-2 protein and increased Apaf-1 protein expression. These data suggest that genistein therapy may enhance the chemosensitivity of melanoma patients.

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Effect of Imedeen Time Perfection active ingredients on collagen I and fibrillin deposition in an *in vitro* skin equivalent

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Imedeen Time Perfection (ITP) is a skin nutritional supplement containing BioMarine complex, vitamin C, grape seed extract and tomato extract containing 10% of lycopene. BioMarine complex with vitamin C and zinc was shown to increase density of dermis and overall quality of skin in clinical tria1(1). In this study, we investigated effects of ITP on the extracellular dermal matrix using in vitro reconstructed skin equivalent (SE). The SE was cultured according to standard laboratory method(2). A porous dermal sponge was seeded with living human fibroblasts. The fibroblasts colonised, proliferated and synthesized extracellular matrix components. After 3 weeks, keratinocytes were seeded on the top and gradually built a fully developed epidermis. The ITP active ingredients were added in the culture medium at early stage of the SE development. Effect of the ingredients was examined after 6 weeks of supplementation using morphological and immunohistochemical methods (staining of elastin, fibrillin, colla-gen type I, III, IV and VII). The SE was shown to closely resemble native human skin both morphologically and regarding expression of characteristic biomarkers(3). Our new results demonstrate that treatment of SE with ITP stimulates formation of even more in-vivo-like dermis. Histological results showed that ITP had a strong positive effect on the dermal reconstruction by stimulating fibroblast colonisation, proliferation and deposition of extracellular matrix components in the porous structure of dermal substrate. The immunohistological results showed a marked increase of collagen type I, the major component of extracellular matrix, and fibrillin, the major component of microfibrils of elastic tissue.

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Effects of pimecrolimus on dendritic cell functions

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Pimecrolimus is a novel calcineurin inhibitor of the ascomycin family. It has been shown to inhibit the production of inflammatory cytokines by T cells and mast cells in vitro, and to inhibit cutaneous inflammation when applied systemically or topically to the skin. However, pimecrolimus does not inhibit the sensitization phase of allergic contact dermatitis, and shows only low potential for affecting other systemic immune responses. Since calcineurin inhibitors, such as cyclosporin A and tacrolimus, have been shown to also affect dendritic cell (DC) function, we tested whether pimecrolimus also modifies the differentiation of DC from murine bone marrow precursors. DC were generated by culture of bone marrow (BM) cells in the presence of 150 U/mL GM-CSF and 75 U/mL IL-4 for 6 days, followed by DC activation with 20 ng/mL LPS or 1 μg/mL CD40L. Pimecrolimus was added either at the beginning of the BM culture (Day 0) or at the timepoint of DC activation (Day 6). Cells were harvested and analyzed at Day 8 of culture. Cell yield was not affected when pimecrolimus was added at the timepoint of DC activation (Day 6 of the BM culture). The morphology of DC that developed from bone marrow precursors was normal and DC surface molecules (CD11c, CD40, CD80, CD86, I-A) were largely unaffected by the presence of pimecrolimus, when added either at the onset of BM culture or at the time of DC activation. However, we observed a modest increase in CD86 expression and a decrease in CD54 expression upon exposure with pimecrolimus. Apart from a slight upregulation of TNFàand IL-6 release, cytokine production by DC was also not inhibited by the presence of pimecrolimus in doses of up to 50 ng/mL. Thus, pimecrolimus suppressed neither inflammatory cytokines nor the production of IL-12 and IFNã, and it also did not induce IL-10 production by DC. In conclusion, pimecrolimus does not inhibit the differentiation and activation of bone-marrow-derived dendritic cells in vitro.

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Effect of pimecrolimus, tacrolimus, cyclosporin A, betamethasone 17-valerate, dexamethasone, and hydrocortisone on cytokine production and T-cell proliferation induced in human peripheral blood mononuclear cells by anti-CD3 monoclonal antibody

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Pimecrolimus is an ascomycin macrolactam derivative specifically designed and developed to treat inflammatory skin diseases. It has been shown to be highly effective and safe in atopic dermatitis after topical administration, and in chronic plaque psoriasis after oral application. T-cell activation and the release of inflammatory cytokines play a key role in inflammatory skin diseases, such as atopic dermatitis and psoriasis. In this study, we compared the inhibitory activity of pimecrolimus with tacrolimus and cyclosporin A, as well as with the corticosteroids betamethasone 17-valerate, dexamethasone and hydrocortisone, on anti-CD3 monoclonal antibody-stimulated cytokine production (TNFα, IFNγ, GM-CSF, IL-1β and IL-8) in human peripheral blood mononuclear cells (PBMC). The rank order of potency is (range of IC50 values for the measured cytokines): Pimecrolimus (0.30-0.77 nM) ~ tacrolimus (0.12-0.22 nM) ~ betamethasone 17-valerate (0.32-0.63 nM) ~ dexamethasone (0.99-3.4 nM) > cyclosporin A (6.3-9.3 nM) > hydrocortisone (29-44 nM). We also compared the inhibitory activities of these compounds on T-cell proliferation in the same system. The potency of T-cell inhibition (average IC50 of three independent experiments) is: Tacrolimus (0.18 nM) > pimecrolimus (1.1 nM) ~ betamethasone 17-valerate (1.7 nM) ~ dexamethasone (2.5 nM) > cyclosporin A (11 nM) > hydrocortisone (100 nM). In conclusion, pimecrolimus inhibits the anti-CD3-stimulated release of inflammatory cytokines from PBMC with a similar potency to tacrolimus, whereas tacrolimus appears to be more potent in suppressing T-cell proliferation. The corticosteroids betamethasone 17-valerate and dexamethasone have a similar activity to pimecrolimus, and a greater potency than cyclosporin A.

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Pimecrolimus shows, in contrast to tacrolimus and cyclosporine, only marginal effects on immunization by oral administration in a rat model

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The calcineurin inhibitors pimecrolimus (PI), cyclosporin A (CsA) and tacrolimus (TA) were compared in a 4-week oral rat study investigating the immune system's potential to respond to immunization with keyhole limpet hemocyanin (KLH). Groups of 10 male Wistar rats were treated once daily with 10 or 30 mg/kg PI, 3 mg/kg TA or 20 mg/kg CsA placebo. Five animals from each group were immunized with 50 µg KLH in adjuvant on Days 16 and 24; the other five served as non-immunized references. Macroscopic and microscopic aspects of the KLH-challenge site, KLH-specific serum Ig titers, and lymphocyte phenotypes in blood, spleen and draining lymph nodes were investigated. The evaluation of the granuloma formation at the KLH challenge site revealed a suppression of the peripheral immune response in the following order: CsA > TA >PI = control. The formation of KLH-specific antibodies (IgM and IgG) was fully inhibited in CsA-treated animals to the level of non-immunized controls. KLH-specific IgM titers were similar in immunized controls, PI- and TA-treated animals. A slight to moderate reduction of IgG titers with PI at 30 mg/kg and a slight to severe reduction with TA at 3 mg/kg was observed. No effect on KLH-specific antibody production was seen with PI at 10 mg/kg. Flow cytometric investigations of the lymphocytes in the lymph node draining the KLH challenge site indicated a similar moderate decrease in total CD3+, CD3+/CD4+ and CD3+/CD8+ T lymphocytes for PI at 30 mg/kg, TA and CsA, whereas these changes were marginal for PI at 10 mg/kg. In peripheral blood, the KLH-induced increase in total white blood cells and CD3+ T cells was evident with PI and to some extent with CsA, but not with TA, when compared with the respective treated non-immunized reference groups. In conclusion, the capacity to respond to the antigen was not affected with PI at 10 mg/kg. Only a slight effect with PI at 30 mg/kg was observed, whereas the functional inhibition of the immune system was shown to be more pronounced with TA at 3 mg/kg and more so with CsA at 20 mg/kg.

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The combination of bexarotene capsules and photopheresis induces clinical response in patients with mycosis fungoides

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Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL), a malignancy of skin-homing T-cells that affects more than 1200 persons annually in the USA. Therapies are directed to the stage of disease. Both extracorporeal photopheresis (ECP) and bexarotene (Bx), a novel RXR selective retinoid, are effective treatments for MF. Median time to Bx response is 4 months (dose of 300 mg/m2/day) and 6 months for ECP. Bx has been shown to induce apoptosis of CTCL cell lines in vitro. ECP works through the induction of T-cell apoptosis. The combination of Bx-ECP may allow for the use of lower doses of Bx and provide faster time to response than for either treatment alone. To investigate the efficacy of combination Bx-ECP therapy, 6 CTCL patients (stages IB-IVA) were enrolled in a study of low-dose Bx (75-225mg/day) with ECP. Patients began with low dose Bx for 7 days and subsequently also received ECP every three weeks for a total of 4 treatment cycles. Efficacy was assessed at regular intervals up to 90 days, when the study was terminated. Weighted skin scores showed a substantial improvement in less than 2 months after treatment initiation with significant improvement at the end of the trial ($p<0.001$, Friedman test). Grade 2 toxicity (incr. trigl.) was seen in 1 patient. To determine treatment effect on apoptosis, lesional skin biopsies obtained at baseline, day 7 (Bx alone), and end of study (Bx and ECP) were processed for TUNEL reaction. In 2 patients, TUNEL positive cells increased substantially between baseline and end of study. One stage III patient with the most dramatic induction of apoptosis, continued to maintain clinical improvement, with treatment, 8 months after study termination. Our data show that combination therapy of Bx-ECP significantly improves cutaneous manifestations of CTCL and shortens the response time as compared to each treatment modality alone. Apoptosis of T-cells in lesional skin could be used to predict clinical response to treatment.

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Influence of intravenous immunoglobulin therapy on autoantibody titers to beta4-integrin in patients with mucous membrane pemphigoid

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Mucous membrane pemphigoid (MMP) is an autoimmune mucocutaneous blistering disease characterized by the presence of circulating autoantibodies. Recently, patients with MMP have been described to have autoantibodies to beta4 integrin. Objective: the objective of this study was to prospectively analyze, over a 18-months period, the influence of intravenous immunoglobulin (IVIg) therapy on autoantibody titers to b4-integrin in a group of 13 patients. Methods: levels of autoantibodies to b-4 integrin were measured by specific immunoblot assay, at monthly intervals. Results: prior to initiation of IVIg therapy, the sera of 13 patients demonstrated the presence of high autoantibody titers to b-4 integrin. A statistically decline in the autoantibody titers to b-4 integrin was observed after five months of receiving the first cycle of IVIg therapy. This gradual decline in autoantibody titers continued until patients were observed to have non-detectable titers to b-4 integrin after 14 months, of receiving IVIg therapy. Once patients achieved non-detectable titers, these patients were considered to be in a serological remission. This serological remission was sustained for an additional four months of observation. Conclusion: autoantibody titers to b-4 integrin can be used to monitor the serological response to treatment in patients with MMP. Patients with severe MMP, who are treated with IVIg therapy, as described in our protocol, achieve a long-term serological remission.

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The use of N-acetyl glucosamine to replace alpha hydroxy acids in skin care

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Alpha hydroxy acids have been used to treat skin for both dermatological and cosmetic problems. Though there are many known benefits of the use of alpha hydroxy acids on skin, there have been recent reports that topical treatments with alpha hydroxy acids increase skin damage resulting from UVB. Additionally, high concentrations of alpha hydroxy acids have also been found to cause an irritating effect. In order to find alternatives to alpha hydroxy acids, we investigated a variety of amino sugar compounds that were previously reported to inhibit the reaggregation of dissociated corneocytes by modulating cellular adhesion. *In vivo*, we observed that topical treatments of product containing 10% N-acetyl glucosamine led to an increase in skin moisturization, decrease in skin flakiness, and the normalization of stratum cornuem exfoliation. *In vitro*, we observed an upregulation of differentiation markers, keratin 10 and involucrin, in keratinocytes treated with N-acetyl glucosamine. CD44 is a lectin cell adhesion molecule that is also expressed in keratinocytes. Amino sugars such as N-acetyl glucosamine may competitively bind to CD44, modulating keratinocyte cellular adhesion. We hypothesize that these amino sugars modulate keratinocyte cellular adhesion and differentation, leading to the normalization of stratum corneum exfoliation. We propose the use of amino sugars such as N-acetyl glucosamine as alternative compounds to replace the use of alpha hydroxy acids in skin care.

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Human keratinocytes treated with damaged RNA have enhanced resistance to UVB and elevated DNA repair

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Ultraviolet irradiation of normal human keratinocytes induces a cytotoxic effect. The chromophore for this effect is believed to be genomic DNA. However, DNA damage can be repaired in UVB irradiated keratinocytes. Previous work by our group and others suggest that host cell DNA repair can be triggered by internal as well as external, damaged DNA. Therefore another possible sensor of UVB exposure and damage to DNA may be the RNA molecule. To test this hypothesis we investigated the effects of UVC damaged RNA on keratinocyte cell survival and DNA repair. Treatment of HaCaT keratinocytes with UVC irradiated rabbit globulin mRNA pre- and post- UVB exposure increased the survival of the HaCaT cells. The increased survival correlated with reduced DNA damage as measured by the number of thymidine-thymidine dimers. Addition evidence supporting the ability of damaged RNA to enhance host cell DNA repair is provided by the observed effects of damaged RNA on host cell reactivation of DNA repair. In these experiments we observed that external rabbit globulin mRNA enhanced repair of a plasmid inserted into the host 3T3 cells. This repair is further enhanced if the mRNA is first pre-irradiated with UVC. The above evidence suggests that damaged mRNA can trigger a host cell DNA repair pathway.

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Influence of intravenous immunoglobulin therapy on autoantibody titers to BP Ag 1 and BP Ag 2 in patients with bullous pemphigoid

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Bullous pemphigoid (BP) is a subepidermal autoimmune blistering disease which is characterized by blisters on the skin. Autoantibodies to components of the basement membrane zone are usually observed the sera of patients with BP. Autoantibodies to the bullous pemphigoid antigens, (BP Ag 1, 230 kDa desmoplakin protein) and (BP Ag 2, 180 kDa hemidesmosomal protein), are present in the sera of BP patients. In this study, we report the influence of IVIg therapy on autoantibody titers to both BP BP Ag 1 and BP Ag 2 in 14 patients with bullous pemphigoid. In this prospective study, we measured autoantibody titers to both BP Ag 1 and BP Ag 2, over a period of 18 consecutive months on each patient, using an immunoblot assay. Prior to the initiation of IVIg therapy, the sera of 11 patients demonstrated the presence of high autoantibody titers to both BP Ag 1 and BP Ag 2. Three patients had autoantibodies to BP Ag 1 only. A statistically significant decline in the autoantibody titers to both BP Ag 1 and BP Ag 2, was observed after four months of receiving the first cycle of IVIg therapy. This gradual decline in autoantibody titers continued until patients were observed to have non-detectable titers to BP Ag 1 after 11 months, and to BP Ag 2 after 10 months of receiving IVIg therapy. Once patients achieved non-detectable titers, these patients were considered to be in a serological remission. This serological remission was sustained for an additional seven months of observation. Autoantibody titers to BP Ag 1 and BP Ag 2 can be used to monitor the serological response to treatment in patients with BP. Patients with severe BP, who are treated with IVIg therapy, as described in our protocol, achieve a long-term serological remission.

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Intravenous immunoglobulin therapy in patients with epidermolysis bullosa acquisita refractory to conventional therapy

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Epidermolysis Bullosa Acquisita (EBA) is a chronic autoimmune blistering disease. EBA can be very frustrating to treat since patients with EBA can be refractory to conventional therapy. In this study, intravenous immunoglobulin (IVIg) therapy was used in two patients with EBA who were refractory to conventional therapy. Both patients received a total IVIg dose of 1-2 mg/kg per cycle. The parameters used to assess the response to IVIg therapy included time observed for effective control of disease, total duration of IVIg therapy, number of IVIg cycles, systemic drug therapy, and frequency of recurrences and relapses. The differences in the following variables were statistically analyzed using the SAS UNIVARIATE software running the two sided Wilocoxon signed rank test pre- and post-IVIg therapy: number of side effects, frequency of recurrences and relapses, duration and total dosage of prednisone therapy, and the quality of life. An effective clinical response to IVIg therapy was observed in both patients. The two patients were able to discontinue all previous systemic therapies. The difference between Pre- and Post-IVIg therapy were statistically significant for the number of side effects, frequency of recurrences and relapses, duration and total dosage of prednisone, and the quality of life. One patient achieved a sustained and prolonged clinical remission and is off all systemic therapies. The other patient continues to receive IVIg as monotherapy at increased intervals while maintaining an effective clinical control of their disease. IVIg improved the quality of life in all patients and demonstrated a steroid sparing effect. IVIg therapy can induce a long-term clinical remission in patients with EBA unresponsive to conventional agents.

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Side effects of IVIg therapy in patients treated with long-term IVIg therapy in autoimmune mucocutaneous blistering diseases

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IVIg therapy has been recently reported in the treatment of patients with pemphigus vulgaris (PV), mucous membrane pemphigoid (MMP), and bullous pemphigoid (BP). The incidence of side effects secondary to IVIg therapy has been reported to be low. In this study, we report the side effects of intravenous immunoglobulin therapy in, 26 patients with PV, 20 patients with MMP, and 10 patients with BP, who had been non-responsive to conventional therapy. A retrospective analysis was done to analyze the side effects of IVIg in a large case series of patients with PV, MMP, and BP and PV. The following information was recorded in this study: demographics, dose of IVIg therapy, number of cycles of IVIg therapy, duration of IVIg therapy, the side effects of IVIg therapy, associated symptoms of side effects, the duration and frequency of side effects, and prevention and treatment of side effects. There were 26 males and 30 females and the mean age of onset of disease was 60 years. All patients received an IVIg dose of 1-2 gm/kg/cycle. The mean number of cycles patients received was 18 over a period of 22 months. The most common side effect was headache (24), flu like symptoms (12), chills and rigors (5), nausea and vomiting (4), palpitations (2), and brochospasm (1). The duration of symptoms would be from about 1 to 4 days. The headaches were prevented in all 24 patients with premedication with intravenous salumedrol (40-60 mg) and acetaminophen (650 mg). In conlcusion, we observed a low incidence and frequency of side effects of IVIg therapy in patients with PV, MMP, and BP over a long-term follow-up. IVIg is a relatively safe therapy in the treatment of autoimmune blistering diseases.

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Topical retinol improves fine wrinkles of natural/chronological aging

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Like all organs, skin ages with the passage of time. Evident in upper inner arms of the elderly, naturally aged skin is thinner, laxer and more finely wrinkled than in younger skin. These features plus dyspigmentations are more severely manifested in photoaged skin. Since topical retinoic acid (RA) improves photoaged phenotype, we hypothesized that similar features of natural aging would also respond to the retinoid. Topical retinol (ROL) is metabolically converted to RA in human skin, and causes less skin irritation than RA. Therefore, we investigated the effectiveness of ROL in ameliorating clinical features of skin chronoaging. Thirty-six elderly subjects in two senior homes were enrolled in a left/right comparison study of 0.4 % ROL versus its vehicle. Mean age was 87 (80-96), and male to female ratio was 1:2.5. Up to three times weekly treatments were administered by our staff for 6 months. Punch biopsy was performed from both sides at baseline and at the end of treatment. Unoccluded application of our 0.4% ROL lotion to normal human skin induced retinoid responsive gene, CRABP-II, mRNA by 3 fold ($p<0.05$; n=5), indicating its ability to penetrate human skin and cause retinoid pharmacology. Of the 36 subjects, 23 completed the study. Fine wrinkles were assessed clinically using a semiquantitative 10 point score (0=none, 9=most severe). At baseline, clinical severity between the left and the right upper inner arms were similar. ROL treatment significantly reduced the fine wrinkles from 7.3±4.9 at baseline to 4.9±0.2 at week 24, where as vehicle treatment had no effect ($p<0.0001$; n=23). The improvement was associated with a 40% increase in glycosaminoglycan (GAG) expression ($p<0.05$; n=6). Topical ROL was well tolerated by the elderly subjects. Mild erythema and scaling was observed with ROL treatment. In conclusion, topical ROL improves fine wrinkles associated with natural aging. Significant induction of GAG, which is known to retain substantial water, may be one cause of the wrinkle effacement.

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Ultraviolet A1 is an effective antifibrotic phototherapy for scleroderma, and sclerodermoid varient of chromic graft-versus-host disease

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Dermal fibrosis in scleroderma (SD) and sclerodermoid graft-versus-host disease (SD-GVHD) is indistinguishable. Despite differences in pathogenesis, the fibrosis common to both conditions is a result of dermal collagen increase (greater synthesis and/or reduced degradation). We have previously reported that ultraviolet (UV)-B/A2 (290-340nm) irradiation induces matrix metalloproteinases (MMPs) and simultaneously inhibits procollagen synthesis in normal human skin *in vivo*. Compared to UVB/A2, UVA1 (340-400 nm) is minimally erythemogenic and therefore is safer to use on patients. We investigated the response of SD (n=5) or SD-GVHD (n=6) patients to UVA1. UVA1 (65 or 130 J/cm^2) irradiation was given 3 times a week for up to 16 weeks. The degree of skin hardness (induration) was assessed clinically using a modified Rodnan score (mRS: 0=none, 3=severe). The mean mRS of target area at baseline was 2.4 and 2.0 for SD and SD-GVHD, respectively. At the end of treatment, there was 32% ($p=0.05$) and 37% ($p=0.04$) reduction in the mean mRS for SD and SD-GVHD, respectively. Procollagen I and III, transforming growth factor-β and connective tissue growth factor mRNA levels were substantially elevated in involved skin, compared to uninvolved normal skin. UVA1 treatment markedly reduced the elevated levels of these gene transcripts, while inducing those of MMPs (-1, -3, and -9). Total collagen content of the sclerotic skin, as measured by hydroxyproline, was also significantly reduced with UVA1. In SD-GVHD, UVA1 efficiently depleted tissue infiltrating T cells (CD3$^+$) present in the lesional skin. All subjects tolerated UVA1 phototherapy without sunburns or other signs of phototoxicity. In conclusion, UVA1 is an effective antifibrotic treatment for SD and SD-GVHD. We speculate that it will be beneficial in other fibrotic conditions where collagen homeostasis has been perturbed to produce a net increase in this matrix molecule.

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Mechanisms of lovastatin-induced apoptosis in melanoma cell lines

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The cholesterol lowering medication lovastatin inhibits cellular proliferation and induces apoptosis in various tumors and is associated with decreased melanoma incidence in a double-blinded, placebo-controlled, clinical trial for coronary disease. We investigated how lovastatin affects human melanoma cell viability in vitro. Lovastatin caused cytotoxicity in human and murine melanoma cells while at the same doses, lovastatin induced little toxicity in an epidermoid carcinoma cell line A431. Lovastatin-induced cell rounding, increased the percentage of apoptotic cells in human melanoma cells A375, and resulted in high levels of active caspase 3 in A375 cells indicating that lovastatin induced apoptosis in human melanoma cells. Supplementation with geranylgeranyl pyrophosphate completely blocked the lovastatin effect in A375 cells, while farnesyl pyrophosphate supplementation only slightly blocked the lovastatin effect. These data suggest that inhibition of geranylgeranylation is probably the predominant mechanism of lovastatin-induced apoptosis in human melanoma cells. We also examined the effects of lovastatin on the expression of several apoptotic regulatory proteins including Bcl-2, Bax, IAPs etc. Preliminary data suggest that lovastatin down-regulated expression of inhibitor of apoptosis protein 2 (IAP-2) in A375 cells. In summary, this study shows that lovastain induces human melanoma cell cytotoxicity by a geranylation specific apoptotic mechanism in A375 melanoma cells, and provides further evidence for the possible role of lovastatin as a therapeutic/ chemoprevention agent for melanoma.

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Pharmacokinetic and safety analysis of a topical cyclosporine A prodrug (CGC2001)

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Oral cyclosporine A (CsA) is effective in treating psoriasis, but its clinical utility is limited by its side effects. Topical CsA could overcome these adverse effects, but it is ineffective due to poor skin absorption. The objective of this study was to perform a pharmacokinetic (PK) and safety analysis for a topical CsA prodrug (CGC2001), where CsA was conjugated to an oligo-D-arginine via a pH-sensitive linker. When CGC2001 was applied to the backs of rats, full thickness skin penetration of CsA was observed one hour after drug application using CsA specific antibody staining. Maximal staining was observed around 6 hours and the majority of the drug was cleared from the dose site after 24 hours. Similar kinetics were obtained using free CsA concentrations determined by LC/MS/MS. At each time point up to 24 hours, the total CsA (free CsA plus CGC2001) was higher than the free CsA levels in skin, suggesting some CsA was cleared as an intact conjugate. This observation was consistent with the 1:4 ratio of free CsA to CGC2001 found in fecal samples. When CGC2001 4% Ointment was applied, the free CsA and total CsA skin concentrations were 1.0 and 5.2 ng/mg tissue respectively at 8 hours. This free CsA was comparable to the CsA skin levels (0.8 ng/mg tissue) obtained after oral dosing with 3 mg/kg CsA for 7 days, indicating that we were able to deliver therapeutic levels of CsA via topical application of CGC2001. A study using radiolabeled CGC2001 in rats indicated that 24% of applied drug penetrated the skin over 8 hours. There was no detectable CsA/CGC2001 in plasma or other organs. Approximately 99% of the absorbed drug excreted in feces and less than 1% was found in urine in 48 hours. There were no observable adverse effects when 50 mg and 375 mg of CGC2001 4% Ointment were applied twice day for 90 days to rats and rabbits, respectively. These data indicate that topical application of CGC2001 is safe and provides therapeutically relevant local concentrations of CsA in skin while avoiding systemic exposure and toxicity.

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Optimal use of extracorporeal photopheresis in cutaneous T cell Lymphoma

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Extracorporeal photopheresis (ECP) is an apheresis procedure incorporating ultraviolet-A irradiation of photosensitized peripheral blood mononuclear cells approved for cutaneous T-cell lymphomas (CTCL) mycosis fungoides and Sezary syndrome, characterized by monoclonal expansion of malignant helper T cells. Our previous prospective study of ECP monotherapy [JAAD 35: 573, 1996] is compared to a retrospective analysis of ECP/combination therapy. Eighty-six CTCL patients were treated at our institution with ECP monotherapy or combination therapy between 1988-2002. They included 48 males and 38 females, 70 Caucasians, 8 Blacks and 8 Hispanics with a median age of 64.5 (38-81). Fifty-four patients receiving ECP monotherapy achieved an overall response rate (RR) based on improvement in body surface area involved of 43% (23/54). Four were stage IA-IIA (RR=75%, 2 CR, 1 PR); 50 were stage III-IVB (RR=40%, 5 CR, 15 PR). There were 7 complete remissions, and two were > 3 years duration. Patients received a median of 21 ECP sessions (range 5-40+) over a median of 13 months (range 1-35). The median time to response was 5 months (range 1-32 months). An additional 32 patients received ECP in combination with other active systemic therapies (psoralen ultraviolet A, interferon, oral bexarotene or isotretinoin, denileukin diftitox, mustargen, or total body electron beam irradiation). Patients received a median of 14 ECP sessions (range 3-54+) over a median of 14.5 months (range 1-72). The median time to response was 5 months (range 1-36 months). They achieved an overall RR of 56% (18/32) including 4 at stage IA-IIA (RR=50%, 1 CR, 1 PR and 28 at stage III-IVB (RR=57%, 3 CR, 13 PR). Side effects were limited to interferon seizure (1 pt) and catheter related sepsis (4 pts). Other mild symptoms included headaches, nausea, transient increased erythema, and acral edema. ECP has a higher response rate when combined with other conventional treatments.

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Pimecrolimus lacks the strong apoptosis-promoting activity of corticosteroids in human dendritic cell cultures

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We and others have reported previously that corticosteroids (CS) profoundly inhibit many aspects of dendritic cell (DC) biology, such as DC differentiation, maturation and the function to stimulate primary T cells. In contrast, pimecrolimus, a new drug for the treatment of inflammatory skin diseases, does not affect DC differentiation or function. We were prompted by the finding that CS applied topically to murine skin mediated the apoptosis of epidermal Langerhans' cells in vivo (see Abstract W Hoetzenecker et al., SID 2003). In this study, we focussed on human dendritic cells (DC) and their myeloid precursors as targets of CS-mediated apoptosis. Differentiation of DC from CD14+ monocytes was induced by GM-CSF + IL-4 in the absence or presence of different concentrations of pimecrolimus or CS (dexamethasone or beta-methasone-valerate). The proportion of apoptotic cells was measured before and during cytokine-mediated DC differentiation on days 1, 2, 3, and 6 by FACS following annexin-V, 7-amino-actinomycin D (7-AAD) and TUNEL staining. CS induced apoptosis in a time- and dose-dependent fashion. At 100 nM, CS induced apoptosis of approximately 25%, 50% and 85% of all cells on days 1, 2 and 3 of culture, respectively, while 10 nM of CS induced apoptosis of approximately 30% on any of the first 3 culture days. In sharp contrast, less than 5% TUNEL-positive and less than 15% annexin-V-positive cells were present at any time point in monocyte-DC cultures in the presence of 100 nM pimecrolimus, and this was similar to the degree of apoptotic cells found in the controls. Taken together, in contrast to pimecrolimus, CS induce apoptosis of myeloid-type DC and this may contribute to their unfavourable safety profile upon prolonged use.

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Treatment with oral pimecrolimus significantly improves atopic dermatitis with a clear dose-response effect

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Pimecrolimus is a non-steroid, selective, inflammatory-cytokine inhibitor. It has been shown to have high skin-selective anti-inflammatory activity with a low potential for affecting systemic immune responses. The results of a study to evaluate the efficacy and safety of pimecrolimus for the treatment of moderate to severe atopic dermatitis (AD) are presented here. In a double-blind, placebo-controlled, parallel-group, dose-finding study, 103 adult patients with moderate to severe AD were randomized equally to receive either placebo, 10, 20 or 30 mg po pimecrolimus bid. The study consisted of a pre-treatment phase, 12 weeks of double-blind treatment and a 12-week post-treatment phase. Efficacy was assessed by EASI and the percentage of patients who were clear or almost clear of disease as defined by the investigator's global assessment (IGA). The median change of the EASI score from baseline showed a dose-dependent effect and was greatest for the 30-mg bid treatment group. At 12 weeks, the median percentage reduction of EASI was 68%, 48%, 37% and 19% in the 30 mg-bid, 20 mg-bid, 10 mg-bid and placebo groups respectively. Following treatment with the highest dose of pimecrolimus, 27% of patients were clear or almost clear of disease at 12 weeks. In the two highest dose cohorts, more than half of the patients had not experienced disease relapse (defined as the time when active therapy for AD is started) 4 weeks after stopping treatment with oral pimecrolimus. Oral pimecrolimus was well tolerated throughout the study. Pimecrolimus was effective in the treatment of AD providing dose-dependent and prolonged disease control. Based on its efficacy and convenience, pimecrolimus has the potential to become a systemic treatment of choice for the management of moderate to severe atopic eczema.

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T cell-mediated internalization and clearance of a murine antibody specific for the psoriasis drug target CD11a

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Efalizumab is a humanized monoclonal antibody specific for the human antigen CD11a that is currently in phase III clinical trials for the treatment of moderate to severe psoriasis. The α-chain component of the leukocyte function-associated antigen-1, CD11a, is involved in T-cell activation and trafficking into the dermis and epidermis through interaction with intercellular adhesion molecules. Efalizumab inhibits T-cell activation and trafficking, and appears to be efficacious in the treatment of psoriasis. Fundamental to our understanding of the pharmacology of efalizumab is a detailed knowledge of its clearance from the body. In humans, the relationship between CD11a expression and serum levels of efalizumab has been mathematically described by a CD11a-mediated clearance model for efalizumab. However, although hypothesized, no experimental data is available as yet to explain how the interaction with CD11a results in the clearance of efalizumab. To explore pathways for drug clearance, we employed the murinized monoclonal antibody muM17, specific for mouse CD11a. muM17 was shown to have a pharmacokinetic profile and functional characteristics similar to efalizumab in humans. Using this antibody, we hypothesized that muM17 would be internalized by murine T-cells both in vitro and in vivo leading eventually to the clearance of muM17 in vivo. Initial studies to elucidate the cellular fate of the internalized antibody were also conducted. Results from these studies indicate that muM17 is internalized by isolated murine T-cells in a time-dependent manner. Similarly, the antibody was internalized in vivo by peripheral mononuclear blood cells and the spleen. Nearly 75% of the internalized antibody was cleared from the cells by 24 hours in vivo. Future studies using both efalizumab and muM17 will continue to focus on elucidating 1) the role of CD11a in antibody internalization, 2) the molecular mechanism of antibody internalization, and 3) the sub cellular localization and fate of the internalized antibody both in vitro and in vivo.

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Protection of experimental bullous pemphigoid by IVIG is mediated through the IgG protection receptor FcRn

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Intravenous immune globulin (IVIG) has been used in the treatment of inflammatory and autoimmune diseases. Numerous mechanisms have been proposed to explain the mode of action of IVIG. In the present study, we used a murine model of bullous pemphigoid (BP) to test the hypothesis that the effect of IVIG in autoantibody-mediated skin bullous diseases is to accelerate the degradation of pathogenic IgG through the FcRn. BP is a subepidermal blistering disease associated with autoantibodies directed against two hemidesmosomal antigens, BP230 and BP180. The experimental mouse model of BP is induced by passive transfer of rabbit anti-murine BP180 (mBP180) antibodies into neonatal mice. Using this BP animal model we have demonstrated that skin disease severity is directly correlated with the circulating anti-mBP180 antibody levels. FcRn transports IgG and is responsible for maintenance of serum IgG levels. We found that administration of IVIG to C57BL/6J mice prevented subepidermal blistering triggered by pathogenic anti-mBP180 antibodies. This protection is associated with a significant reduction of circulating pathogenic IgG. The protective effect of IVIG depends on FcRn activity because total anti-mBP180 IgG levels at 24 and 48 h post-injection were almost the same in FcRn-deficient mice with and without IVIG treatment. These data demonstrate that IVIG treatment leads to increased FcRn-dependent clearance of pathogenic IgG; therefore, modulation of FcRn function represents a therapeutic target for autoantibody-mediated bullous diseases.

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Pimecrolimus does not affect cytokine and chemokine secretion and adhesion molecule expression in primary human keratinocytes and dermal fibroblasts

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Pimecrolimus has been shown to inhibit the production and release of inflammatory cytokines in T cells at nM concentrations, and to prevent the release of pro-inflammatory mediators from mast cells. In the skin, keratinocytes and fibroblasts can modulate T-cell and mast-cell responses by the synthesis of cytokines and chemokines, as well as adhesion molecules. In this study, we investigated whether pimecrolimus affects functions in primary human epidermal keratinocytes (KC) and human dermal fibroblasts (HDF), in comparison with the glucocorticoid dexamethasone. Pimecrolimus had no effect on the induction of GM-CSF, IL-6, IL-8 (CXCL8), MCP-1 (CCL2), MIP-3a (CCL20), IP-10 (CXCL10) and RANTES (CCL5) in KC and HDF, and on eotaxin (CCL11) in HDF at concentrations up to 10 mM. The expression of the adhesion molecules ICAM-1 (KC and HDF) and VCAM-1 (HDF) was also unaffected. In contrast, the glucocorticoid dexamethasone inhibited IL-6, IL-8, eotaxin and GM-SCF in HDF at IC50 concentrations ranging from 0.5 to 19 nM. In conclusion, no effect was observed with pimecrolimus in keratinocytes and fibroblasts. This confirms the high cell selectivity of pimecrolimus, acting on T cells and mast cells.

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Bacterial quorum-sensing molecules: potential utility in the treatment of inflammatory skin disease

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N-acyl homoserine lactones (AHLs) are a series of signalling molecules employed by gram-negative bacteria to co-ordinate gene expression with population density, thereby acting as 'quorum-sensors'. Recent data has indicated that AHLs also interact with the mammalian immune system, resulting in immunosuppressive and anti-proliferative effects. Such compounds might be of utility in the treatment of hyperproliferative and inflammatory disease such as psoriasis and we were therefore interested to determine whether a series of AHLs had growth modulatory effects on keratinocytes. Subconfluent normal human keratinocytes were cultured in the presence of increasing concentrations of three AHLs, two (OdDHL and OtDHL) with long carbon side chains (C12 and C14 respectively) and one (OHHL) with a shorter, C6, side chain. Both OdDHL and OtDHL induced a dose-related suppression of keratinocyte growth over 24 hours at concentrations as low as 3.125 μM when cell number was assessed by an MTT assay. Moreover, 3H incorporation was completely inhibited at 25μM by both OdDHL and OtDHL. OHHL was without effect in either of the assays. To assess potential toxicity of the AHLs, keratinocytes were cultured in increasing concentrations of the three compounds for 24 hours and LDH release was assayed colorimetrically. No toxicity was seen from any AHL compound at concentrations up to 50mM, though at higher concentrations a dose-related increase in LDH release was observed with both OdDHL and OtDHL. Again, OHHL was without effect. These data indicate that long chain bacterial AHLs have profound effects on keratinocyte growth and proliferation at non-toxic doses. This observation is of relevance to the effects that gram-negative bacteria (for example Pseudomonas aeruginosa) have on the skin, for example non-healing cutaneous ulceration, but also suggests a potential therapeutic utility of these compounds in inflammatory and hyperproliferative skin diseases such as psoriasis.

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Safety profile of oral pimecrolimus in atopic eczema and psoriasis: a pooled analysis from two dose-finding studies

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Pimecrolimus, a cell-selective inflammatory cytokine inhibitor, has been developed specifically for the treatment of inflammatory skin diseases. A pooled safety analysis is presented from two dose-finding studies performed with oral pimecrolimus. 243 adult patients were included in the pooled safety population of the randomized, double-blind, placebo-controlled, parallel-group, dose-finding studies of patients with moderate to severe atopic eczema or psoriasis. In both studies, patients were randomized to receive either placebo or oral pimecrolimus 10, 20 or 30 mg bid in a 1:1:1:1 ratio for 12 weeks. Safety assessments included monitoring and recording of adverse events, regular monitoring of laboratory assessments (haematology, blood chemistry, urinalysis) vital signs and physical examination assessments. Oral pimecrolimus was well tolerated. Less than 3% of patients withdrew from the study due to adverse events. The only adverse event that showed a clear dose-effect relationship was a transient, generally mild to moderate feeling of warmth. Headache and common gastrointestinal symptoms such as nausea and dyspepsia were the most commonly observed adverse events. No effect of treatment on blood pressure was observed in any of the study groups. No significant changes in mean serum creatinine and glucose tolerance were observed in any group.Evaluation of skin immune response to skin-recall antigens before and after treatment showed that the skin cellular immune response to bacterial and fungal antigens was preserved during treatment with oral pimecrolimus. Oral pimecrolimus was shown to have an excellent safety profile in the short-term treatment of psoriasis and atopic eczema. This conforms with pharmacogenomic data from a previous study in psoriasis. Following further evaluations in long-term studies, pimecrolimus has the potential to become a drug of choice for the systemic treatment of inflammatory skin diseases.

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The efficacy, safety, pharmacokinetics, and pharmacodynamics of efalizumab in patients with plaque psoriasis: results of phase III trials

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Studies evaluating efalizumab, a humanized monoclonal IgG_1 antibody, have consistently demonstrated that efalizumab provides benefit for patients with plaque psoriasis. Efalizumab blocks the interactions between leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), which inhibits the activation of T cells and interferes with trafficking into the dermis and epidermis. The effects of efalizumab therapy were evaluated in placebo-controlled phase III trials followed by an open-label trial. During the randomized, double-blind, placebo-controlled trial, subjects with moderate-to-severe plaque psoriasis received 1.0 mg/kg/wk subcutaneous (SC) efalizumab or placebo for 12 weeks. Upon completion, subjects who had received efalizumab were enrolled into the ongoing open-label follow-on study and received an additional 12 weeks of SC efalizumab 1.0 mg/kg/wk, for a total of 24 weeks of continuous efalizumab therapy. The Psoriasis Area and Severity Index (PASI) responses observed for the 556 subjects in the study are consistent with response rates observed previously; pooled data from 2 prior phase III trials demonstrated that 29.2% of 394 subjects treated with efalizumab 1.0 mg/kg/wk had ∝75% PASI improvement at Day 84. The pharmacokinetics and pharmacodynamics of efalizumab were characterized, and overall, subjects received adequate efalizumab exposure. The data from these studies are to be finalized and presented in detail. The results of these phase III trials are consistent and provide evidence for the clinical benefit of efalizumab in the treatment of patients with plaque psoriasis.

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Efficacy, safety, and pharmacokinetic outcomes observed in subjects with plaque psoriasis during long-term efalizumab therapy: results from an open-label trial

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Efalizumab, an anti-CD11a humanized monoclonal IgG_1 antibody, blocks leukocyte function-associated antigen-1/intercellular adhesion molecule-1 (LFA-1/ICAM-1) binding, inhibiting T-cell mediated events in the pathogenesis of plaque psoriasis. The effects of 1 to 2 years of continuous efalizumab therapy for this chronic disease are being evaluated in an ongoing, open-label, multicenter trial. Subjects with moderate-to-severe disease received 2.0 mg/kg/wk subcutaneous (SC) efalizumab for 12 weeks. At Day 84, subjects with ≥50% improvement in Psoriasis Area and Severity Index (PASI) or Overall Lesion Severity (OLS) of Mild, Minimal, or Clear were eligible for maintenance efalizumab therapy. Maintenance treatment (MT) consisted of SC efalizumab 1.0 mg/kg/wk At the conclusion of the initial 12 weeks of treatment, 41% and 81.1% of the 339 subjects demonstrated ≥75% and ≥50% PASI improvement, respectively. Preliminary analysis of 6 months of therapy showed that 77.1% of 288 subjects maintained ≥50% PASI improvement and 50% of these subjects had ≥75% PASI improvement. Mean trough levels measured at the end of each 12-week MT period were: MT-1 14.5 μg/mL; MT-2 15.0 μg/mL; MT-3 14.6 μg/mL; and MT-4 15.6 μg/mL. MT is ongoing; additional data for this 12-month cohort will be presented. Common adverse events were headache, nonspecific infection (eg, colds), chills, pain, nausea, asthenia, and fever. Consistent trough levels measured during MT show no evidence of efalizumab accumulation; therefore, no pharmacokinetic changes are observed with long-term efalizumab treatment. The efficacy outcomes suggest that long-term efalizumab therapy maintains PASI improvement and is well tolerated.

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Pimecrolimus induces neuropeptide release at early stages of topical application *in vivo*

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Primary afferent neurons play an important role in mediating inflammation, pain and pruritus in the skin. The calcineurin inhibitor pimecrolimus (PI) is highly effective in treatment of inflammatory dermatoses, like atopic dermatitis. Due to an unknown mechanism, at the start of topical treatment, PI may transiently induce a feeling of warmth or mild burning. Therefore, we examined whether PI is able to stimulate neuropeptide release in normal mouse skin and in a model of experimentally induced toxic contact dermatitis of mouse skin. Group 1: Application of pimecrolimus cream 1% on normal mouse ears. Group 2: Application of cream (vehicle). Group 3: Benzalkonium chloride was used to induce irritant contact dermatitis (ICD) on right mouse ears. At various timepoints (30 minutes, 2 hours, 6 hours) pimecrolimus cream 1% was applied, and mouse ears harvested for ELISA (SP, CGRP) after 30 minutes. Group 4: Vehicle control to Group 3. Left ears without treatment were used as additional controls. Expression of neuropeptide (neurokinin) receptors were quantified from mouse ears 2—8 hours after treatment by quantitative RT—PCR. Our data show that pimecrolimus cream 1% enhances the release of SP and CGRP in a time-dependent manner during the early phase after PI treatment of mouse ears. Protein concentrations for SP were slightly increased in the ICD model indicating higher release of SP in inflammatory skin compared with controls. In contrast, no quantitative changes were observed in neuropeptide receptor expression after treatment at all timepoints investigated. Transient early side-effects of PI may be due to stimulation of neuropeptide release from afferent sensory nerve fibers at early stages of topical treatment. In addition, neuropeptides such as SP and CGRP may contribute to the anti-inflammatory capacity of PI. Whether this is a direct or indirect effect and whether long-term treatment with PI results in a decreased release of cutaneous neuropeptides is currently under investigation.

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Quantitation of cutaneous cytokine mRNA levels following anti-IL-12 psoriasis therapy: identification of a psoriasis subset

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Inhibition of IL-12 may downregulate type I cytokines. Therefore, cutaneous cytokine mRNA levels were evaluated in 18 psoriasis patients (pts), with at least 3% BSA, who participated in a multicenter, nonrandomized, open-label, phase I study to assess the safety and efficacy of single, intravenous infusions of a human monoclonal antibody to human IL-12 (0.1-5mg/kg). Clinical efficacy was assessed by PASI. Total RNA isolated from plaque biopsies obtained at −24h, 48h and 2wks post infusion was evaluated for cytokine expression by real time RT-PCR, normalized by 18s ribosomal RNA. At baseline, TNFα, RANTES, IL-10 and IFNγ mRNA levels correlated (Pearson correlation) within and among themselves. Additionally, type I cytokines in the IL-12 cascade (ie, IL-12p40, IFNγ, TNFαand subsequent inflammatory cytokines IP-10, RANTES, IL-8), also correlated. By contrast, MCP-1, IL-18 and IL-12p35 were not observed to correlate. Across all groups, pts who achieved > 75% improvement in PASI at 16 wks, had significantly decreased expression of IL-12p40 and TNFαat 2 wks (n=12, p=0.04, each). The level of IFNγ mRNA decreased by 58±4.8% in all patients at 2wks ($p<0.005$). This is consistent with anti-IL-12 interference of type I immunoreactivity within lesions and with the notion that efficacy of anti-IL-12 therapy is associated with inhibition of type I cytokines. Decreased expression of mRNA for these cytokines preceded clinical improvement. Interestingly, across all groups, pts who achieved > 75% improvement in PASI at 16wks (n=12) had higher average baseline TNFα mRNA levels than pts who achieved ≤75% improvement in PASI (n=6)($p <0.05$). Thus, baseline TNFα mRNA levels may identify a novel psoriasis subset and predict therapeutic responsiveness, particularly at lower doses.

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Atorvastatin inhibits metastasis of human melanoma cells *in vivo*

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Rho GTPases are signaling molecules known to control cell motility. Several recent studies have suggested a role for Rho proteins in mediating tumor metastasis independent of their effects on cell proliferation. Since Rho proteins require post-translational modification with a geranlygeranyl moiety for full activity, we tested the effect of blocking geranylation on localization, downstream signaling and stimulation of invasion. Statin drugs, which deplete the cell of cholesterol through inhibition of HMG-CoA reductase, have pleiotrophic effects on many isoprenylation events within the cell. We hypothesized that statin treatment could prevent Rho activation and thus Rho-mediated metastasis. We developed a luciferase based in vivo imaging model to visualize A375 cells metastasizing in SCID mice in real time. Our results demonstrate that statin treatment inhibits Rho activation in vitro and furthermore, that oral administration of atorvastatin to SCID mice dramatically inhibits the metastatic dissemination of RhoC overexpressing A375 human melanoma cells in vivo. These results suggest that statin treatment may serve as a prophylactic regimne against metastasis of melanoma.

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Inhibition by levocetirizine of VCAM-1 expression on human dermal endothelial cells

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Local tissue eosinophilia is a well recognized feature of allergic inflammation and adhesion molecules play a potent role in such eosinophil recruitment. Cetirizine has been shown to inhibit the accumulation of eosinophils in the skin of pollen sensitive subjects as well as to modulate VCAM-1 expression in atopic dermatitis skin. In the present study, we determined whether levocetirizine - a new antihistamine - inhibited the stimulated upregulation of VCAM-1 expression on human dermal endothelial cells (HDMEC) in response to cytokines in vitro. Since the regulation of adhesion molecule expression - including VCAM-1 - involves the nuclear factor kappa B (NF-kB) translocation, we also evaluated the possible effects of levocetirizine on the TNFalpha-induced NF-kB activation in HDMEC. HDMEC, obtained from normal adult skin, were grown in medium M199 supplemented with 20% fetal calf serum, endothelial growth factor supplement, and antibiotics. Subconfluent Cells were treated with levocetirizine (10-7 to 10-4 M) during 24h and stimulated with 10U/ml TNFalpha for 4h. Expression of VCAM-1 was assessed by FACS analysis. NF-kB activation was measured by electrophoretic mobility shift assay (incubation of nuclear extracts with a 32P-end-labeled oligonucleotide probe containing a binding site for NF-kB; use of positive and negative probes and supershift assays with antibodies to the p50 and p65 subunits). Levocetirizine inhibited the VCAM-1 expression induced by TNFalpha at the surface of HDMEC in a dose-dependent fashion, from 10-4 to 10-7M. Although there was no basal level of NF-kB activation in HDMEC alone or under levocetirizine treatment, TNFalpha induced a strong activation of NF-kB which was significantly inhibited by levocetirizine according to a dose-dependent manner. These data support previous works on anti-inflammatory effects of levocetirizine and suggest a possible mechanism whereby it may inhibit eosinophil accumulation in vivo.

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Identification of oral pemphigoid antibody binding site(s) in human a6 integrin molecule

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Oral pemphigoid (OP) is a subset of mucous membrane pemphigoid (MMP) with disease limited to oral cavity. Recently we demonstrated that, sera from OP patients recognized a 120 kDa protein. This binding was blocked by monoclonal antibody to human a6 integrin. This protein was further characterized as human a6 integrin. The purpose of this study was to identify specific site(s) on human a6 integrin molecule to which sera from OP patients bind and determine its role in the process of blister formation. We cloned the fragments representing the extracellular and intracellular domain of a6 molecule from normal human gingival mRNA into an expression vector and mapped the region to which sera from OP patients bind by western blot analysis. We have attempted to determine the role of the immunodominant region in pathogenesis by demonstrating the ability of the rabbit antibody to the immunodominant region to produce separation of the epithelium from the submucosa when incubated with normal human buccal mucosa in an in vitro organ culture model. Majority of the OP sera tested bound to the N-terminal end of the extracellular domain (EC1.0) of the human a6 integrin. Further sub-cloning of EC1.0 demonstrated that a smaller fragment extending from 23 aa to 131 aa (EC1.1) was responsible for this binding. This region may have multiple antibody binding sites. Antibody to human EC1.1 produced in rabbit, resulted in separation of the epithelium from the submucosa, histologically identical to that observed when normal human buccal mucosal tissue was cultured with OP sera in an human buccal mucosa organ culture model. These observations identify EC1.1 as the antibody binding site for sera of OP patients and suggest a possible role for it in blister formation.

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The proteasome inhibitor "bortezomib" overcomes melanoma chemoresistance *in vitro* and *in vivo*

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Melanoma is well known for its extreme chemoresistance. Multiple studies, indicate that melanoma cells counteract anticancer agents by inactivating programmed cell death (apoptosis) and reinforcing multiple survival signals. We hypothesized that targeting survival and pro-apopototic pathways simultaneously might be an efficient therapeutic strategy in melanoma. The proteasome inhibitor bortezomib (Velcade, formerly known as PS-341) is exhibiting promising effects in clinical trials for several chemoresistant tumors (e.g. multiple myeloma). The cytotoxic action of Bortezomib has been associated, at least in part, with the inactivation of the NF-kB pathway and a still poorly-defined induction of apoptosis. At doses that do not affect the viability of normal melanocytes (i.e. 1-50 nM), bortezomib induced massive cell death (mean viability <10%) of a panel of 24 aggressive melanoma cells resistant to other chemotherapeutic drugs. To test the efficacy of bortezomib in controlling melanoma growht and metastasis vivo, GFP-tagged human melanoma cells were injected either subcutaneously or intravenously, respectively. Those mice were treated with vehicle control or with 1 mg/kg of bortezomib every two days for a period of 2-3 weeks. Whole body fluorescence images indicated that bortezomib had a significant effect in controlling growth and metastasis of melanoma cells ($p<0.01$), which was confirmed by histological analyses. Biochemical studies of melanoma cells treated in culture and in mouse xenograft models indicate that bortezomib is able to activate multiple apoptotic executors (caspases) and bypass negative signals from antiapoptotic regulators. Cell death was also independent of the Ras/BRAF/ERK functional status of the tested cells ($p<0.01$). We consider these results as a proof of principle for the design of new strategies to overcome melanoma chemoresistance in vivo.

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Alefacept modulates type I inflammatory genes in psoriasis

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In psoriasis vulgaris, a Type 1 cytokine genomic pathway is active and defined by increased expression of genes encoding pro-inflammatory cytokines (IL-12, γ-IFN), chemokines (IL-8, MIG), transcription factors (STAT1, IRF-1), and the enzyme iNOS. To better understand the relationship between expression of Type 1 genes, disease activity, and T-cell targeted biologicals, we sequentially analyzed gene expression in skin lesions of 22 patients with psoriasis vulgaris treated with a standard regimen of alefacept. Patients were designated as responders (n=12) or non-responders (n=10) to alefacept based on reductions in epidermal hyperplasia and reversal of keratin 16 (K16) immunohistochemical staining at week 13. Gene expression at weeks 0, 2, 6, and 13 was analyzed by quantitative, RT-PCR. In 12/12 responders, there was a marked reduction in K16 mRNA (mean 7-fold reduction, p=0.003), whereas minimal reductions in K16 mRNA in the non-responders. Untreated psoriasis lesions had increased of Type 1 inflammatory gene expression (IL-12p40, γ-IFN, IRF-1, STAT1, IL-8, MIG, and iNOS) in comparison to uninvolved skin. Responders showed consistent suppression of these mRNAs in week 13 biopsies, whereas non-responders had small changes in expression of these key inflammation-related genes. We used a novel multivariate statistical approach to assess the relationship of disease improvement to simultaneous changes in several Type 1 pathway genes. There was a high correlation (r=0.759) between disease response and gene expression in Type 1 inflammatory pathway genes. These data establish a central role for T-cells in regulating a complex inflammatory pathway in psoriatic lesions.

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Topical corticosteroid activity in human skin is associated with single nucleotide polymorphisms in three genes involved in alcohol metabolism

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Betamethasone dipropionate, 0.05% in ointment without branched alcohols produces highly variable baseline skin blanching response (SBR) in Caucasians that can be stratified into three major response cateogories by reflectance colorimetry: High, Medium and Low, representing 20%, 35% and 45% of the population (n=26), respectively. The branched alcohols propylene glycol (PG) and isopropanol (IPA) considered inactive excipients in high potency corticosteroid products alter SBR as a function of baseline SBR category ($p < 0.05$) independent of skin drug content. Betamethasone dipropionate in ointment with PG decreases SBR 2X in High responders, but increases SBR 2X and 6X in Medium and Low responders to the drug, respectively ($p< 0.05$ ANOVA). In contrast, IPA in the lotion product has no effect on High and Medium responders, but increases SBR 6X in Low responders. When IPA and PG are combined in the lotion, IPA

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Evaluation of risk factors for fogo selvagem: case-control study

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Foao Selvagem (FS) is an autoimmune blistering disease endemic in Brazil. A population based case-control study of FS was conducted in an Amerindian Terena community located in Midwestern Brazil. The focus of this case-control investigation was to evaluate the extent to which aspects of housing and hygiene, life activities, family and social relationships, health history, animal and other exposures. and HLA type/status contribute to the occurrence of FS in an environment of high endemicity such as Limao Verde. The following studies were conducted using a specially designed survey form. A single interviewer (VA) obtained the information which was entered in a database for further analysis. All FS cases (n=30) and controls (community controls n =108; family controls n= 90) were selected by matching on gender, age, race and occupation. A 13-page questionnaire was administered to each patient and control, and included a final assessment of the house and the living conditions of patients and controls made by the interviewer. The highlights of this study are here described: 1-demographic characteristics: 228 individuals, mean age of 40 years old; there was no predominance of gender among the population studied-about 70% of the participants belong to the Terena ethnic group, and over 60% of the interviewees were engaged on farming activities;2-exposure to bed bug bites were higher in FS patients (63.3%) than controls (community=32.4%/ family=34.4%),3-exposure to kissing bugs were higher in FS patients (53.3%) than controls (community=30.6%/family=34.4%); 4-FS patients usually live in poorer houses, made of adobe with thatched roofs, in comparison to controls. These results suggest that the environmental antigen (s) triggering the autoimmune response in FS may be linked to exposure to hematophagous insects.

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Assessment of knowledge, habits and attitudes of high-school adolescents towards skin cancer risk factors, sun exposure, sun effects and sun protection measures in Porto Alegre - Brazil

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Adolescence has been identified as a high risk period for unprotected sun exposure, sunburns and induction of skin cancer, the most prevalent malignancy in the world, as well as the most preventable. This study examines patterns of sun protection and sun exposure among Brazilian adolescents and their knowledge about the effects of the sun. Methods: During July-November, 2002, a total of 720 adolescents aged between 12 and 19 were recruited from a random sample of 25 high-schools in Porto Alegre - Brazil. The volunteers completed a validated 40 questions survey that provided information on sunscreen use, sunbathing habits, tanning bed use, and cutaneous solar effects. Results: About 99% of the adolescents have already heard about UV light hazards, and the top source of sun protection information was the midia (89,6%), while schools and physicians contributed with only 15% and 23,1%, respectively. Although 85% of the students pointed skin cancer as a risk of sun exposure, and 25,6% answered that sun exposure brings absolutely no benefits, only 47,6% referred frequent sunscreen use during summer time, and only 3% during the winter. The great majority (75,6%), answered that people look better with a tan. Among those who referred frequent use of sunscreens, 21,3% choose sunscreens factors less than 15 and 63,7% do not reapply them regularly. Tanning bed use was referred by 3,5% of the students and they were more likely to be female (76,9%). Sunburns with bullae were reported by 18,6% of the respondents (60,4% of the female sex) Conclusions: These results suggest the need for increased education about skin cancer prevention habits. Students seem to be well informed about the risks of sun exposure, but are not practicing correctly the sun protection measures. Because the media is a major information source, and it is well known by its influence on the promotion of tanning, it is important to ensure that messages about sun risks and sun protection are correct. Physicians and schools should be more engaged on teaching people about the effects of the sun. This study will be important in defining the aims of the educational campaigns on skin cancer in Brazil.

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A randomized controlled clinical trial on the efficacy of green tea extract in reducing UVB-Induced erythema at 2xMED

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Background: Chronic exposure of solar ultraviolet (UV) radiation has several adverse biologic effects on the skin. Protective clothing and use of sunscreens are not sufficient to protect our skin against the harmful effects of the solar UV radiation. Several studies have shown that green tea extract affords significant skin photoprotection. Objective: This study aims to determine the efficacy of a green tea cream (10%. w/w) in reducing UVB-induced erythema. by comparing it to placebo and sunblock. Adverse effects from the application of the extract are also investigated. Methodology: Thirty subjects included in the study. On day-I. minimal erythema dose (MED) determination was done. Dav-2. baseline ervthema of each of the six test sites on the back was determined using a mexameter. then seventy milligrams of sunbiock. green tea and placebo. were randomly applied, each on two test sites on the lower back; one site was exposed to UVB at 2xMED thirty minutes after application of the creams, the other site was not irradiated. Twenty four hours after irradiation, mexameter readings were recorded, and clinical grading of erythema was done. Results: Comparison of means of erythema indices and clinical grading showed that the mean average of placebo exposed to UVB is significantly different from means of green tea and sunblock exposed to UVB ($p = 0.00000114$. and $p = 2.74 \times 10$ respectively), and that difference between green tea and sunblock exposed to UVB was not significant (Duncan Test for Multiple Comparisons). Five subjects (17%) developed irritant contact dermatitis to the green tea cream. Conclusion: Seventy milligrams of green tea extract (10%. w/w) containing 65% catechins applied on 2 cm x 2 cm (4 55 rng/2cm of catechins) is significantly effective in reducing UVB (2xMED)-induced erythema compared to placebo. Its inhibition of UVB-induced erythema is comparable to a sunscreen. Irritant contact dermatitis developed in 1 7% of subjects

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Gene *p16* ink 4A in malignant melanoma- study of a family

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Melanoma is a malignant tumor potentially curable if diagnosed early in it's biological course.
In familiar melanoma, mutations of the tumor suppressor gene TP16 /p16 ink4A, in cromossome 9p21, have been described and related to predisposition to the development of melanomas within families. With the objective of identifying patients with this predisposition, we studies 8 individuals of family with melanocytic lesion (melanocytic nevi, displastic nevi and /or melanoma). We searched for point mutations in portion 3' éxon 2 gene *p16* ink4A in the DNA from skin biopsies of the studies patients. In two of the samples, the DNA analysis from the skin lesion (In this case, melanocytic nevi), Showed a different mirgration pattern from the simple strand of genomic DNA extracted from peripheral blood lymphocytes. This finding suggests the existence of genetic alterations specific of the gem cells line, which would predispose this patient to the development of malignant melanoma.

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Eritema nodoso in the Hospital Nacional Dos de Mayo from 1990-2001

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Erythema nodosum (EN) is an acute, nodular, eryhematous eruption that usually is limited to the extensor aspects of the lower legs. EN is presumed to be a hypersensitivity reaction and may occur in association with several systemic diseases or drug therapies, or it may be idiopathic. The inflammatory reaction occurs in the panniculus. The purpose of the present retrospective study was to evaluate the principal causes of erythema nodosum in the Hospital Nacional Dos de Mayo, Lima-Perú, in a period of 11 years, between 1990 and 2001. 104 patients have been studied. All patients have clinical and hispathologic–septal panniculitis-diagnosis of erythema nodosum. The female: male ratio was 4: 1 and the mean age was 31 years. We found a cause in 46.15% cases, the most frequent cause of erythema nodosum was an infectious agent, including beta-hemolytic strptococcus (32.69%). In 53.85% the etiology of erythema nodosum remained undetermined and were qualified as idiophathic . The clinical characteristic presentation of erythema nodosum was founded in 95.19%.

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Chronic hydroarsenicism (CHA) is the prolonged exposure to arsenic (As) species in drinking water

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Chronic hydroarsenicism (CHA) is the prolonged exposure to arsenic (As) species in drinking water. In 1997 we diagnosed a 10 year-old (y/o) female from Zimapán, Mexico with CHA who eventually died of related complications. Therefore we investigated urinary arsenic (UAs) levels in Zimapán, and whether or not more children were affected. A correlation between UAs levels and cutaneous markers of CHA in children was sought. Method: Our study was comparative, transversal, observational, and prospective. Subjects were randomly selected from 4 different communities where two acted as controls and two as exposed cases based upon historic as levels in water. The 17 y/o and younger were included and examined by two dermatologists looking for cutaneous features of CHA. Urine samples were analyzed for as content using a method previously described. (1) Results: 296 controls and 263 cases were selected. Of these, 139 (47%) controls and 126 (48%) exposed were children aged 2-17 y/o. Abnormal levels (higher than 50 mg/l) were encountered in 92 (73%) of the cases and 8 (6%) of controls. Average UAs levels in unexposed communities were significantly lower 19+/-18 mg/l than those encountered in exposed ones 665+/-636 mg/l ($p < 0.00001$). Cutaneous lesions encountered in controls and exposed subjects were respectively: Hyperkeratoses 0% vs. 20.9%, hyperpigmentation 0% vs. 12.2%, and confetti-like pigmentation 0% vs. 2.6%, all reaching statistical significance. ($p<0.001$) The two youngest children with high UAs levels (57 and 193 mg/l) were 2 y/o. The highest level encountered was 2860 mg/l in a 13 y/o female, this patient however, had no cutaneous lesions. The youngest patient with lesions (hyperkeratoses) was a 6 y/o female (UAs levels 33 mg/l). Conclusions: CHA has been reported worldwide.(2-4) However, to our knowledge, CHA has only been reported in children in Chile,(5-6) Taiwan,(7) Argentina(8), Thailand,(9) Bangladesh,(2,4), and Comarca Lagunera, Mexico.(10-11) As levels were never as high as those here reported. A correlation was found between cutaneous signs and UAs levels. Skin manifestations are believed to take years to appear, however we found them in children as young as 6 years of age. This could mean either exposure to high As levels since early childhood or even in-utero. This hypothesis would be supported by the fact that high levels of UAs have been reported in children and newborn babies, who had normal looking skin.(2) One case of CHA-related squamous cell carcinoma has been reported in a 13 y/o Mexican patient.(11) None of our patients had malignant lesions. CHA in Zimapán implies a severe risk for As-related complications that may be prevented through a clean water supply.

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Benign cephalic histiocytosis: two case reports

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Benign cephalic histiocytosis is a rare, self-healing, non-Langerhans cell histiocytosis, characterized by a popular and macular eruption mainly located on face and cheeks. It was first described by Gianotti et al in 1971 and until now, 40 cases have been reported. We report two patients, an 8 and 22 month-old boys, with a 2 and 10 month course, consisting on an asymptomactic, slightly raised, round, brownish papules on the face and shoulders. Oral mucosa was spared and there was no evidence of lymphadenopathy or hepatosplenomegaly. For both patients haematologic blood test and X-ray of skull were normal. The skin biopsy for both specimens revealed a histiocytic infiltrate with few multinucleated gaint cells with peripheral nuclei. The lesional cells were CD1a and S-100 negative in the 8 month-old boy. Ultraestructural studies were performed on both patients, and comma-shaped intracytoplasmic structures were found in histiocytes of the second patient, confirming the diagnosis. As benign cephalic histiocytosis is a self-healing entity, with no systemic associated disease, no treatment is needed beside regular medical follow-up

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Chinese herbal medicines influence the follicle biology in vitro and in vivo

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It is more than thousand year histories to treat hair diseases with Chinese herbal medicines in China. But what component play the important role are still not very clear. With an organ culture model of mouse vibrissa follicles we investigated the length of hair growth, morphological change of the bulb, and 3 TdR incorporation in the follicles that were cultured with the decoctions of 55 Chinese herbal medicines, The results demonstrated that the hair growth were significantly stimulated in the follicles cultured with ligustrum lucidum (A), angelica dahurica (B), bletilla striata reichb (F), schizonepeta tenuifolia (B), astragalus mombranaceus (B), astragalus complanatus (R), glycyrrhizic acid repectively ($r<O.O5$). These results were supported with the data obtained from the tests of 3H-TdR incorporation. Further more, the experiment also found that ligustrum lucidum (A), angelica dahurica (B) and bletilla striata reichb (F) deceased catagen formation and reduced the number of apoptosic (TUNEL positive) cells in mouse hair follicles in in vivo, when mice were fed with the decoctions of the herbs. In order to make the insights into the specific molecular mechanisms of the Chinese herbs, suing the approach of RT-PCR, we investigated the mRNA expressions of HGF and VEGF in follicles which were cultured with the Chinese herbs. The results show that at day 4 of culture the steady-state levels of HGF and VEGF were significantly higher in the herbs treated mouse vibrissae follicles than that in vehicle-treated controls. We demonstrate that some of these 55 Chinese herbal medicines stimulates hair growth of normal mouse vibrissae hair follicles or skin hair follicles by stimulating proliferation and inhibiting apoptosis of hair follicle cells in vitro or in vivo, and that it effects mRNA expression levels of HGF, \7EGF, which might contribute to the clinically observed hair growth-promoting effects of the Chinese herbal medicines. These results have furnished the information to develop new medicines for hair disorder.

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Nitrogen-containing biphosphonates inhibit cell cycle progression in human melanoma cells

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Cutaneous melanoma is one of the most malignant human tumours, due to hits high metastatic potential and its resistance to classical chemotherapy regimens. Therefore, new therapy alternatives for melanoma are needed. Biphosphonates are potent inhibitors of bone demineralization, widely used for the treatment of osteoclast-mediated resorptive bone disorders, including tumor-induced osteolysis osteolysis. Recent studies suggest that bisphosphonates may also have a direct antiproliferative and proapoptotic effect in different types of human tumor cells. The inhibition of the mevalonate pathway and, consequently, of the prenylation of signalling proteins such as the small GTPases may explain this antineoplasic action in the case of the subclass of nitrogen-containing bisphosphonates. The present study compared the in vitro effects of bisphosphonates with different mechanism of action and different antiresorptive potency on human melanoma cell lines. The non-aminobisphosphonate clodronate and two amino -bisphosphonates, pamidronate and zoledronate were analyzed. We could show that the amino -bisphosphonates inhibited the proliferation of melanoma cells and altered the cell cycle progression, causing accumulation of the cells in the S phase of the cell cycle. These effects were dose-dependent. Amino -bisphosphonates also induced apoptosis in the melanoma cell lines in dose-and time-dependent manner. In contrast, the nonamino-bisphosphonate clodronate showed no effect on proliferation, cell cycle progression and apoptosis induction in melanoma cells. These findings prove a direct antitumoral effect of bisphosphonates in melanoma cells it/ vitro and further support the hypothesis of different intracellular mechanism of action for amino- and non-amino-bisphosphonates. Our data suggest that amino -bisphosphonates may be a promising alternative for the adjuvant therapy of melanoma.

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Influence of age of the patient, type of the disease and site of the procedure on outcome of epidermal grafting in vitiligo

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The success of epidermal grafting may be influenced by various factors which have not been studied to date. The objective of the study was to determine the influence of age of the patient, location of vitiligo patch and type of the disease on the outcome of the procedure in our patients and in data derived from literature analysis. Epidermal grafting was performed in patients with stable vitiligo. All published studies of SBEG in vitiligo involving 10 or more patients were included in analysis. Procedure was performed in 143 patients. However, the sufficient length (6-months) of follow-up was available in only 117 patients; only these patients were included. Only limited information was available about various factors in majority of the published studies. The success rates for vulgaris disease in the present study and literature were 53% (C.I. 42-64) and 91% (C.I. 81-100) and for segmental/focal disease 61% (C.I. 46-76) 88% (C.I. 82-94) respectively. The success rates in patients aged <20 years in present study and literature were 82% (C.I. 67-97) and 100% respectively and in <21 years were 58% (C.I. 48-68) and 66% (C.I. 56-76) respectively. There was no significant difference in the success rates on different body sites. Among adverse reactions, hyperpigmentation in 32%[24-40] and 17%[14-20], infection in 6%[2-10] and 0%[0-0], and contact dermatitis in 1%[0-3] and 1%[0-2] patients were observed in present study and in analyzed literature respectively. The results were significantly better in segmental/focal vitiligo than in vulgaris, and in individuals <20 years of age than in >20 years of age. However, unlike in medical therapies, the site of treated vitiligo patch did not influence the treatment outcome.

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Use of sunscreens by Brazillian universitarian students

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Several authors have demonstrated an improvement in the cases of skin cancer in the world. The exposure of ultraviolet radiation is considered the main etiologic factor in these lesions. The correct use of sunscreens is one of the methods of protection of skin against the harmful of the ultraviolet radiations. Every tear the Dermatology Brazilian Society realizes a campaign of skin cancer prevention. Our aim was evaluate if Brazilian universitarian students had knowledge about the risks of ultraviolet radiation exposure, through of reduce the solar exposure and improve the daily use of sunscreens.

We interviewed 44 Brazilian universitarian students, asking about the frequency of solar exposure in daily frequency activities, during the practice of exercises or during tanning. We also asked about daily frequency of the sunscreens. The mean age of the students was 22,25. About the frequency of solar exposure in daily activities, 9% answered never, 75% answered sometimes and 16% answered frequently. About the solar exposure during practice of exercises 59% answered sometimes and 41 % answered never. About the intention of tanning 5% answered frequency 65% answered sometimes and 30% answered never. About the daily frequency use of sunscreens 66% don't use anytime, 18% once, 14% twice and 2% three times a day. We conclude that in spite of the Education campaigns the frequency of solar exposure is still high by Brazilian universitarian students and the most of them don't use sunscreens.

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Outcome of mucosal lesions in pemphigus vulgaris treated with conventional or both with conventional and pulse steroids—an analysis of 97 patients

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AIM: Mucosal lesions in pemphigus vulgaris- Response to conventional and pulse steroids. Material and Methods: Mucosal lesions of PV were divided in 2 groups. Pure mucosal pemphigus (Group1) were treated with daily oral conventional steroids whereas mucosal lesions in mucocutaneous variety (Group II) were treated with dexamethasone-cyclophosphamide pulse (DCP) and oral steroids added only if DCP alone failed to control activity of disease. Severity of mucosal disease was graded as follows: Grade I-(<2 oral mucosal areas and no eating difficulty), Grade II- (<2 oral mucosal areas with difficulty in taking solids) & Grade III (< 2 mucosae involved and severe eating difficulties). Treatment continued for 3 month after total healing. Remission defined as free of mucosal lesions for 1 year. Results: Group I had 29 patients & mean duration of mucosal lesions was 6.8 months. Mean dose of oral prednisolone to cure Grade III mucosal lesions was 31.6 mg/day for a mean of 38.8 wks and for Grade II was 25mg/day for 24.1 wks. Group II had 68 patients with mucosal lesions of mean duration of 5.4 months. In Group II, to cure Grade III lesions, in addition to a mean of 12.6 DCPs, mean dose of 25mg/ day prednisolone for a mean of 33.7 wks was required. Grade II lesions in Group II required, in addition to a mean of 10.3 DCPs, a mean dose of 15mg/day prednisolone for a mean of 21.7 wks to heal. Grade I lesions responded to DCP alone. DCP alone failed to treat 9 patients with pure mucosal lesions & shifted to Group I. 14 patients of Group II, treated with DCP alone, had mucosal lesions when DCP was withdrawn, & were treated with prednisolone. Remissions were more and of longer duration in Group I. 40% relapse rate was equal in both groups. Conclusion: Mucosal lesions of moderate to severe intensity (Grade II & III) Respond only to oral prednisolone with or without DCP. DCP alone can cure mild i.e. Grade I mucosal lesions. Daily oral prednisolone is better mode of therapy to treat puremucosal pemphigus.

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Experimental contact sensitivity: a model for both antigen(hapten)-specific and innate immune mechanisms

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Introduction: It was shown that in contact hypersensitivity reaction (CHS) not only antigen-specific but also proinflammatory effects of haptens contribute to the elicitation of CHS, highlighting the importance of mechanisms of innate immunity. In the present study, experimental model of contact sensitivity to dinitrochlorobenzene (DNCB) in inbred AO rats was employed to determine both antigen (hapten)- specific as well as parameters of antigen-non-specific aspects of CHS response. Methods: Ear swelling assay and dermal infiltrate density were employed for quantification of CHS reactions. Draining (axillary and suprascapular) lymph node cell activity and phenotype were determined 72 h following sensitization. Peripheral blood granulocytes activity was determined 24 h following challenge. Resulte: Following local epicutaneous application for 2% DNCB, increased spontaneous, hapten-stimulated and interleukin (IL)-2-driven draining lymph node cell (DLNC) proliferation was detected, reflecting the antigen (hapten)-specific aspect of CHS. At the dose of DNCB employed for the elicitation of CHS in sensitized animals, increased activity of peripheral blood granulocytes (including activation, adhesion and cell survival) was detected. Conclusion: Collectivelly, these data demonstrate the activation of both antigen (hapten)-specific and inmate immunity in CHS reaction, and importance of studying both aspects of reaction in the same animal model.

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The antineoplastic skin protection in patients with psoriasis

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Psoriasis is a chronic recurrent inflammatory dermatosis. The mitotic cycle of epidermal cells in this disease is reduced from 311 to 36 hours and ceratinocytes are formatted 36 times more than in normal skin. The high level of mitosis assists the appearance of mutation cells which even in a healthy organism formed nearly 10mln.every minute. The formation of mutation cell in psoriasis can also rise up after applying for treatment high UVR doses. The first information that skin cancer might be due to light, came from Unna in 1894. Taking into account above mention data it is possible came to the conclusion that psoriasis patients have higher than normal occurrence of skin cancer. However it does not occur. Moreover, psoriasis has fewer carcinomas of the skin than the nonpsoriatics. Paul H. Jacobs (1974) observed that the psoriasis patients were less likely to develop skin cancer than the nonpsoriatics population. Catamnesis, that we performed in 350 patients with psoriasis, who were systematically treated hypererytematous UVR doses from 10 o 30 years in Kiev Dermatology Department (National Medical University), did not reveal any negative effects to the skin. We purposed to determinate the cell and hum oral immunity indexes, that can take part in the antineoplastic protection. The investigations were conducted using a three color flowing cytometry. The immunological examinations we performed in 29 patients with psoriasis revealed higher levels of CD4+(52,16 ± 0,44%vs. 45,09 ± 0.32% in control), CD8+(47,12 ± 0,15% vs. 35,41 ± 0,72 in control), CD22+(25,15 ± 2,02% vs. 14,50 ± 1,32% in control) and NK (CD16) (23,65 ± 0,57 vs. 17,3 ± 0,32% in control). We also revealed higher level of TNF. Taking into account our research and the literature data we can came to the conclusion that in psoriasis antineoplastic protection is formatted in skin by the reason of higher levels of CD4+, CD8+, CD22+, NK and TNF that carry out antineoplastic function in the skin. Therefore using the immunosuppressant drugs in psoriasis can be dangerously, because they suppress antineoplastic function

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Clinical and histopathological characteristics of striae distensae

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Introduction: Striae distensae is a common skin dermatosis that does not cause any significant medical problem. However, they can be a symptom of serious endocrinologic diseases that should be treated by endocrinologists. Striae may be predictors of collagen deficiency, especially in pregnants. Above all, they can be significant distress to those afflicted. The pathogenesis and management are not fully understood. Materials and Methods: We performed a prospective study of 72 men called for military service in Vitbsk Dermatological Hospital aged 23 till 27 during 1999-2002. We studied case histories, performed physical examination, laboratory analysis of blood and urine, made 10 punch biopsies with histopathological examination of atrophic skin using hematoxilin-aeosin and Hart's staining. Results: On physical examination was reveled that striae looked as flattened, thinned skin with a pink hue. 9% has reddish – purple striae. They were generally several centimeters long and 1-100 millimeters wide. Their long axis usually layed parallel to the lines of the skin tension. Histopathological picture was characterized with apoptosis of the epithelium cells. Capillares looked desolate and were absent in some regions. Elastic fibers were destroyed. Sweat glands and hair were solitary and defected. Concomitant skin pathology was revealed in 24 patients (33,3%). Mixed pathology was found in 10 patients (14%). The most frequent skin pathology included- acne (8,3%), palmar and plantar hyperhydrosis (6,9%), tinea pedis (4,2%), pityriasis versicolor (4,2%). It was necessary to consult an endocrinologist in 17 patients (23,7%). The diagnosis of diencephalic syndrome was confirmed in 12 patients (16,7%), Cushing's syndrome in 4 patients (5,6%), using specific laboratory and instrumental examinations. 1 patient (1,4%) used anabolic steroids during 2 months. Conclusion: So we can conclude that striae is not occasional finding and cosmetological problem but an important diagnostic criterion that needs special attention and treatment of accompanying pathology. Histologically striae represent an example of skin atrophy. New methods of treatment and cosmetological correction should be developed to mange patients with extended Striae.

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Gene detection of tetracycline resistance determinant (tetM) and susceptibility determination to antimicrobial agents in Ureaplasma urealyticum

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There is an increasing tendency of Ureaplasma urealyticum (Uu) detection in the patients with chronic and recurrent nongonococcal urethritis (NGU) in China, which may be related to the Antimicrobial resistance. For Uu, tetracycline resistant determinant (tetM) may play an important part in antimicrobial resistance. To determine and compare the MICs of Uu to those 7 kinds of antimicrobial agents with the tetM detection rate, 551 specimens of urogenital discharges from high risk populations of STD were screened for detecting tetM determinants by PCR. A 392 bp fragment corresponding to the tetM determinant was amplified and PCR products were digested by Taq-1 restrictive endonuclease. Also forty-two Uu isolates were subcultured for 3 passages and then incubated with screening agents with a series of concentrations from 64ug/ml – 0.06ug/ml. The MICs were determined after 72-hours. The results showed that the detection rate for tetM was higher (71.4%, 30/42) in Uu isolates than that of high risk populations (36.84%, 203/551). Most positive tetM determinants produced 3 cleaved fragments (190, 130 and 77 bp) after digested with Taq-1 endonuclease. MIC study showed the susceptibility of Uu to Tetracyclines decreased. There were no Uu isolates resistant simultaneously to all 3 antimicrobial agents tested, including tetracyclines, macrolids and quinolones in the study. Taken together, the susceptibility of Uu isolates to the tetracyclines was low and the MIC distribution to antimicrobial agents may be related to the detection rate of tetM determinant. Uu isolates carrying tetM tetracycline-resistant determinant may have a risk of developing drug resistance and should be followed for MIC determination. The special different endonuclease digested patterns of tetM determinants may be existed in some Uu strains.

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Alterations of basement membrane zone in lichen sclerosus

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The aim of the study was to compare alterations of various regions of the BMZ in lichen sclerosus (LS) using laser scanning confocal microscopy. The study included two cases of bullous LS and four cases of non-bullous LS. Three cases of morphea as well as normal human skin were used as controls. Biopsies from patients' skin and a control biopsies from normal human skin were cut into 20 um thick slides and labeled with antibodies against beta-4 integrin (lamina lucida marker), collagen IV and N-terminal end of collagen VII (lamina densa markers) and C-terminal end of collagen VII (lamina densa markers) and C-terminal end of collagen VII (sublamina densa marker) using routine immunofluorescence (IF). Three-dimensional reconstruction of various regions of BMZ showed decreased number and size of the dermal papillae in LS as compared to normal skin. In LS numberous invaginations and holes were present in BMZ on the level of lamina lucida and lamina densa. IF labeling with antibody against N-terminal end of collagen VII - presence of large hole (up to 25 μm) in the lamina densa and revealed the presence of granular material in deep dermis suggesting for partial degradation of lamina densa at the level of anchoring fibers. IF mapping study showed blister formation below the lamina densa. In morphea, flattening of BMZ on the level of lamina lucida, lamina densa and sublamina densa were observed but continuous character of BMZ was preserved. Three-dimensional reconstruction of dermal-epidermal junction in LS revealed alterations of the BMZ, most pronounced at the level of lamina densa and sublamina densa..

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Skin organ culture in the evaluation of contact hypersensitivity reaction intensity

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Introduction: Contract hypersensitivity (CHS) is a local inflammatory response of the skin following challenge of hapten-sensitized animals. The intensity of inflammatory could be quantitated by ear swelling which is the classical manifestation of contact hypersensitivity. In this study-organ culture system was employed to evaluate CHS. Methods: Rat model of contact hypersensitivity to dinitrochlorobenzene (DNCB) was employed. Levels of TNF-α (determined by ELISA) and nitrite accumulation (by Griess assay) were determined in conditioned medium (CM) of ear skin following challenge with DNCB. Result: Dose-dependent increase in TNF-a and in nitrite levels were noted in CM following application of 0.65%, 1.3 % and 2.6% of DNCB to the ears of sensitized rats. The correlation between ear swelling and the levels ($r=0.79$, $p<0.001$) was found. Conclusion: Presented data suggest that this skin-organ system might be employed for evaluation of contact hypersensitivity.

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Analysis of the IgG response to recombinant HSP of 60 kDa from *Streptococcus pyogenes,* human Gro-EL-like and *K. pneumoniae* in psoriatic patients and controls and analysis of the IgG response to the rHSP60 of S. *pyogenes* in psoriatic patients before and during treatment with cyclosporine and its correlation with PASI

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Psoriasis is a cutaneous disease in which the most associated factor in the ethiopathogenesis is streptococcal infections. The presence of IgG antibodies to S. pyogenes recombinant 60kDa heat shock protein (rHSP60Sp) in plaque psoriatic patients has been described. There are no data of the humoral response neither to other rHSP60kDa nor to the rHSP60Sp in psoriatic patients under treatment, nor its association to the degree of severity during their clinical course. Objective: The aim was to measure the humoral response in psoriatic patients and controls to three different recombinant heat shock proteins of 60kDa: S. pyogenes, human, and K. pneumoniae; and determine the levels of IgG antibodies to rHSP60Sp and human GroEL-like in psoriatic patients under cyclosporin treatment in correlation with PASI. Design: Cases and controls and longitudinal study. Material and Methods: By ELISA we measured levels of IgG antibodies to rHSP60 of S. pyogenes, human, and K. pneumoniae in 24 sera of psoriatic patients, 24 sera of healthy controls and 24 of patients with ankylosing spondilytis; and levels of IgG antibodies to rHSP60Sp and human in 100 sera of 28 patients with active plaque psoriasis, under cyclosporin treatment to establish correlation with PASI. Results were analyzed estimating odds ratios with 95% confidence intervals and RR value for the case and controls study and Spearman and Pearson's correlation coefficients for the longitudinal study. Results: Plaque psoriasis patients had significantly higher titers of IgG antibodies to the rHSP60Sp than controls (odds ratio: 17, RR:3.4, 3.7-72.9). The correlation between antibody levels and PASI showed a positive correlation.

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Novel mutation of the ATP2CI gene in aHungarian patient with Hailey-Hailey disease inducded by contract allergens

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Benign familial chronic pemphigus (morbus Hailey-Hailey, HHD) is a rare hereditary condition characterized by development of blisters at sites of friction and in the intertriginous areas. A 57-year-old Hungarian women presenting with clinically typical HHD was studied. The diagnosis was confirmed by skin histopathology. The family history was negative for skin disease. Her skin symptoms started at the age 31 and we speculated whether the skin disease has been induced also by contact allergens. A contact sensitivity to fragrance mix, PPD, iodine, tar, wood-tar was proved by epicutan patch testing. Histology taken from the positive patch test showed a contact dermatitis accompanied by extended suprabasal acantholysis typical for HHD. DNA was isolated from peripheral blood lymphocytes of the proband according to standard techniques. PCR amplification of the entire coding region of the ATP2CI was followed by a heterpoduplex analysis of the 28 amplified exons in a conformation –seneitive 8% acrylamid gel. Bands of altered mobility detected by heteroduplex analysis were directly sequenced and revealed a novel heterozygous nonsense mutation, 1516C-T, Q506X in exon 17 of ATPC1. The presence of the mutation was confirmed in the proband by restriction digestion (Tse I). These new mutations are located in the region encoding the nucleotide-binding (N-) domain of the Golgi Ca2+ ATPase and represent a premature termination codon (PTC). As a consequence, mRNA is probably degraded rapidly by a nonsense-mediated mRNA decay pathway. This study proves that vivo induced pathogenic activity of T lymphocytes leads to pathologic desmosome and keratin network assembly in keratinocytes carrying the Q506X mutation on one allele of the ATP2CI gene encoding a novel calcium pump localized to the Golgi.

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Body site distribution of cutaneous tumours in Chile. Analysis of 4800 tumours from five mayor hositals in Santiago

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The last 10 years have shown an increase of skin cancer incidence rates in Santiago. The purpose of this study was to analyse the anatomical localization of 4,800 shin tumours. For this purpose, 600,000 histophological reports (1992-2001) from 5 hospitals belonging to the Chilean Health Service Program were reviewed. These hospitals covered approximately a population of 2 million people. A total of 568 Malignant Melanoma (MM), 1,021 Squamous Cell Carcinoma (SCC) and 3,212 Basal Cell Carinoma (BCC) were studied. Chi square test was used for the statistical analysis. MM in females was mainly found on legs (23%), cheeks (19.7%) and foot (13.5%); however, MM in male was mainly observed on foot (29.2%), truck (16.5%), arms (16.5%). SCC in females was mainly observed in middle face-area (19.2%), cheeks (16.7%) and genital area (11.8%); SCC in male was also mainly observed in middle face area (12.9%), genital area (11.2%) ears (10.2%). BCC in females and males was mainly observed in the middle face-area (34.1%), cheeks (27.4%), and eyelids (10.3%). Female showed significantly higher number of MM on the cheeks and on the legs than males, however, males showed significantly higher number of MM on the foot. Regarding age, patients over 50 years old showed higher number of MM in the face than those younger than 50 years old ($p<0.05$). Facial MM and facial BCC ($p<0.05$) show increase in the studied decade; however, SCC in the face tend to decline ($p<0.05$). The number of BCC and SCC was significantly higher in the face than in rest of the body, the opposite is observed in MM. Regarding the Breslow Index (BI) m the MMs located in the face, truck, and legs were mainly BI: I; however, MMs located in arms, feet and soles were mainly BI: III and IV. These results are interesting, show concordance and differences with those reported in the literature. Genetics, environmental conditions and lifestyle factors mat explain these findings.

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Efficacy and safety of cyclosporine versus methotrexate in severe psoriasis

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Treatment of patients with severe psoriasis is difficult. From the number of systemic drugs that are available, methotrexate has long been used, but cyclosporine has been recently recommended for the management of severe psoriasis. The purpose of this study was to compare the efficacy and safety of daily cyclosporine with weekly methotrexate in the management of severe psoriasis. Thirty consecutive patients with severe psoriasis were randomly assigned to treatment with cyclosporine or methotrexate. The initial dose of cyclosporine was 3mg/kg/day, which was increased to a maximum of 4mg/kg after two weeks of therapy when the response was not adequate. Methotrexate was administered weekly at a dose of 0.5mg/kg. Clinical response was assessed by calculating PASI score in all patients at biweekly intervals. Patients were followed up fortnightly up to a maximum of 12 weeks. The doses of both drugs were gradually tapered once >75% reduction in disease severity was attained. Marked improvement (>75%) reduction in PASI was noted in all patients except for one in the cyclosporine group. The median time for marked improvement was 5.3 weeks with methotrexate and 6.8 weeks with cyclosporine. Patients on methotrexate were found to have more rapid and complete clearance than those on cyclosporine. Both drugs were well tolerated. Side effects in both the treatment groups were minor, transient, and manageable. At doses with comparable safety profiles, methotrexate resulted in more rapid and cost effective clearance of patients with severe psoriasis. Cyclosporine can provide an effective and safe alternative.

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Combined mutations in keratin 5 and keratin 14 genes causing epidermolysis bullosa simplex-dowling meara

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Most cases of Epidermolysis Bullosa Simplex (EBS) are inherited in an autosomal dominant fashion caused by 1 heterozygous mutation in the genes encoding keratin 5 (KRT5) and keratins 14 (KRT14). However, here we report 2 mutation, E168D in KRT5, Which is a novel mutation, and A413T in KRT14, both occurring in a patient with EBS-Dowling Meara (EBS-DM) and her EBS –affected daughter. We studied a 3 generation non-consanguinous family in which the proband and her daughter had different severities of EBS. The clinical appearance in the proband was more severe when she was a baby but improved significantly with age. The daughter has only blisters on the fingertips and at excoriation sites or the father of the proband's baby who were all clinical normal. A long range3 PCR system was used to avoid amplifying KRT14 pseudogenes and to increase the fidelity of the PCR products. Screening of 50 unrelated normal controls (100 alleles) did not demonstrate either mutation. These mutations are unlikely to be polymorphisms and highly likely to be pathogenic. KRT14-A412T has been reported in EBS-Koebner. Hence, KRT5-E168D could be increasing the effect of KRT14-A413RT resulting in EBS-DM in the proband. However, the daughter's milder phenotype could be due to variable gene expression.

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Regulation of vascular endothelial growth factor expression in human keratinocytes by several antipsoriatic drugs

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Vascular endothclial growth factor is vital in the pathogenesis of psoriasis for the proinflammation and proangiogenesis effects. Keratinocytes is the major resource of VEGF in skin. We investigate if the potent anti-psoriatic properties of tretinoin, calcipotrial, cyclosporine and anthralin are attributed in part to their interference with the regulated VEGF expression by keratinocytes. It was reported that retinoids could down-regulated VEGF expression in cultured human keratinocytes, we examined tretionoin in this experiment as positive control drug. The effect of anti-psoriatic drugs on growth- factor- induced VEGF production by primary keratinocytes were examined at mRNA levels. Both tretinoin and calcipotriol were shown to suppress VEGF mRNA expression in a dose-dependent manner. While cyclosporine had no inhibitory effect on the VEGF production at both mRNA and protein levels. However, the classical anti-psoratic drug anthralin was shown to up-regulate the expression VEGF mRNA, explaining the possibility of irritation when anthralin was applied to skin. Together, these studies identify VEGF down –regulation by keratinocytes as a possible target of some anti-psoriatic drugs action in psoriasis.

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A long-term evaluation in post-burn scars in patients receiving growth hormone

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The hypercatabolic state after massive burns has been effectively treated with anabolic agents, such as human recombinant growth hormone, which stimulate protein synthesis and decrease growth retardation in burned children. Interest in the hypertrophic scar formation became a concern after animal studies showed that growth hormone increased renal scarring. A previous study showed that the treatment of severely burned children with growth hormone during the acute-phase hospital stay did not increase scarring of the burn wound, but the formation of hypertrophic scars, however, are known to occur three to twelve months after injury. The aim of this study was to evaluate patients who received human recombinant growth hormone (0.5 mg/Kg/day) for an increase in the incidence of hypertrophic scar formation compared to patients receiving placebo. A retrospective study was designed to evaluate scarring in patients randomly distributed to receive injections of recombinant human growth factor in the dose of 0.05 mg/kg/day from the discharge date until one year post-burn and patients who received placebo injections. A scar on the face was scored using two different scar scales to evaluate close-up view photographs, taken months 3, 6, 9, 12 and 18 to 24 after burn. The scars were scored by three knowledgeable observers and a mean value calculated for comparisons. No significant difference for the total score of both scales. The evaluation of each parameter was made separately and no significant difference was observed for each parameter. Other ethiopathogenic factors may contribute to the hypertrophic scar formation, and should not be considered a limit for the administration of recombinant growth hormone as a therapeutic approach in burned children.