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REGULATION AND ROLE OF INTERLEUKIN 6 IN WOUNDED HUMAN EPITHELIAL KERATINOCYTES

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Dermal wounding is accompanied by inflammation and the resulting proinflammatory cytokines, including interleukin (IL)-6, are thought to play an important role in the repair process. IL-6 is produced by normal human keratinocytes to various dermatological diseases and we have recently shown it is also required for normal wound repair. However, neither the events responsible for its induction nor its role in repair have been clearly identified. Using a recently developed in vitro wounding model, we demonstrate that IL-6 mRNA is expressed and immunoreactive IL-6 is released from cultures of human epidermal keratinocytes (NHEKs) following wounding. The transcription factors, NFκB and NF-IL-6 (C/EBPβ), which co-ordinately help regulate IL-6 expression, were activated following wounding and preceded the appearance of IL-6. Addition of IL-1α to NHEK cultures increased IL-6 production and activated NFκB and C/EBPβ. Addition of the IL-1α receptor antagonist inhibited both IL-6 mRNA expression and the transcription factors following wounding. Immunoreactive IL-1α was detected in the medium following wounding in the absence of new message. Furthermore, addition of IL-6 to NHEK cultures decreased the expression of keratins 1 and 10, differentiation markers of keratinocytes, while proliferation was not affected. Taken together, these data indicate that constitutive keratinocyte-derived IL-1α is a stimulus for IL-6 production in wounded epidermis, the response involves NFκB and C/EBPβ transcription factors, and IL-6 may be associated with modulation of keratinocyte differentiation rather than proliferation.

The initial reaction following cutaneous wounding triggers the production of inflammatory mediators, including interleukin (IL)-1α, tumor necrosis factor (TNF)-α and IL-6, in the dermis and epidermis from several cell types including keratinocytes. It has been postulated that pro-inflammatory cytokines are not only important for the removal of cellular debris, but are involved in regulating sequential responses after wounding including the formation of a provisional collagen matrix (granulation tissue), re-epithelialization and remodeling.[1,2] Among these proinflammatory cytokines, IL-6 is a major mediator of the host response to tissue injury,[3,4] and is present in wound fluid from mice and humans.[1,2] We reported that impaired wound healing in IL-6 deficient or dexamethasone treated mice could be prevented by administration of recombinant IL-6 or intradermal injection of an expression plasmid containing the full length murine IL-6 cDNA.[5] Consistent with these observations, in situ hybridization of wound tissue from wild-type mice demonstrated that IL-6 mRNA is expressed at high levels in the epidermal keratinocytes at the leading edge of the wound. While IL-6 is not spontaneously produced in intact normal skin, it is induced in the skin in response to bacterial endotoxins, skin irritants, contact allergens, viruses, UV irradiation and thermal damage, suggesting a broad role in dermatotoxic reactions.[6–9]

Neither the role of IL-6 in wound healing, nor the mechanisms responsible for its induction at the wound site, have been well established. While IL-6 has been

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Abbreviations used: IL, interleukin; NF, nuclear factor; EGF, epidermal growth factor; rh, recombinant human; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; NHEK, normal human epidermal keratinocytes

reported to induce keratinocyte cell proliferation in culture,[10] this has not been confirmed in vivo.[11] Furthermore, enhanced keratinocyte cell proliferation is not observed in transgenic mice overexpressing epidermal IL-6, although a thickened stratum corneum is present.[12] More is known regarding the molecular and cellular events responsible for IL-6 induction in keratinocytes. Like most cytokine genes, IL-6 gene expression is regulated by both transcriptional and post-transcriptional mechanisms. Transcriptional regulation is controlled primarily by sequence-specific, DNA-binding proteins referred to as transcription factors. These bind to the *cis*-acting elements of the 5′-untranslated region of the IL-6 promoter and include, among others, NFκB and C-EBPβ (NF-IL-6).[13,14] Using IL-6 promoter deletion analysis, it was demonstrated recently that NFκB and NF-IL-6 binding sites serve as obligatory elements for IL-6 induction in epithelial cells.[15,16] Whether similar factors are responsible for IL-6 induction in epidermal keratinocytes is not known. In the present studies we utilize an in vitro wounding model,[17,18] which allows for reproducible damage to human keratinocytes, to examine the role and events responsible for IL-6 expression in skin wounding.

RESULTS

IL-6 mRNA expression and protein secretion in keratinocyte cultures

RNA was isolated from intact or wounded, growth-arrested, NHEK cell cultures and evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) for IL-6 mRNA expression. Increased IL-6 mRNA expression was detected within 30 min following wounding, peaked at 1–2 h, and returned to control levels by 24 h (Fig. 1A and B). At the 1 h time point, IL-6 expression from wounded cultures was approximately 11-fold greater than that of intact controls (Fig. 1B). Compared to growth-arrested cells, keratinocytes cultured in keratinocyte growth medium (KGM), to allow for proliferation, expressed less IL-6 message following wounding (data not shown). The secretion of immunoreactive IL-6 into the culture medium was examined under similar experimental conditions. As shown in Figure 1C, IL-6 was detected in cell supernatants within 1 h following wounding. IL-6 protein concentrations continued to increase following injury and by 24 h reached a level of 25 pg/ml compared to less than 5 pg/ml in supernatants from intact cell cultures.

NFκB and ClEBPβ activation in wounding

The IL-6 gene contains NFκB and C/EBPβ binding sites within the promoter region that must be occupied for maximal IL-6 expression to occur.[15] To explore whether activation of these cognate regulatory elements are associated with wounding, electrophoretic mobility shift assays (EMSAs) were conducted using probes that represent their consensus binding sites. Within 30 min following wounding, increased NFκB (Fig. 2A) and C/EBPβ (Fig. 2B) binding activity was detected in cells from wounded cultures compared to the intact controls. NFκB and C/EBPβ DNA binding was prevented by the addition of either excess unlabeled NFκB (Fig. 2A, lane 6) or C/EBPβ (Fig. 2B, lane 6) oligonucleotide, respectively, while the respective mutated probes (lane 7) or a non-related oligonucleotide, AP-1 (Fig. 2C and 2D) had no effect on binding activity.

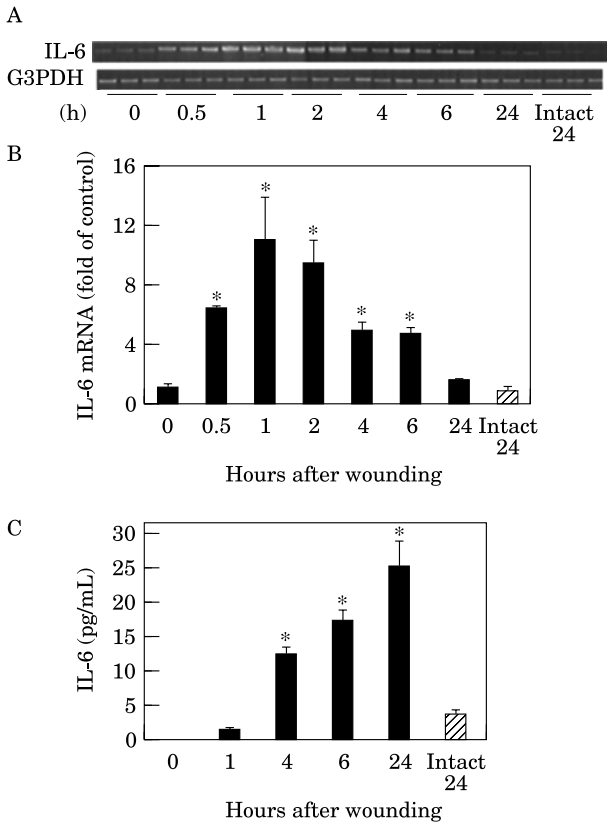

Figure 1. Time course for IL-6 mRNA expression and IL-6 release from NHEKs following wounding.

Sub-confluent NHEKs were pre-incubated in KSM without growth factors for 48 h. The medium was changed to fresh KSM and the monolayer cultures were wounded as described in Materials and Methods. The total mRNA was collected after 0.5, 1, 2, 4, 6 and 24 h after wounding and RT-PCR performed. (A) PCR products were electrophoresed on 2% (w/v) agarose gels and visualized by staining with ethidium bromide. (B) Gels were scanned with a digital image analyzer and the data are expressed as fold-change compared to the intact control (0 h). The density of each amplified cDNA band for IL-6 was normalized relative to that of the corresponding band for G3PDH. (C) The culture media were collected after 0.5, 1, 2, 4, 6, and 24 h after wounding, and IL-6 levels determined by ELISA. Values represent mean ± of three replicate experiments. *$P<0.05$ vs control.

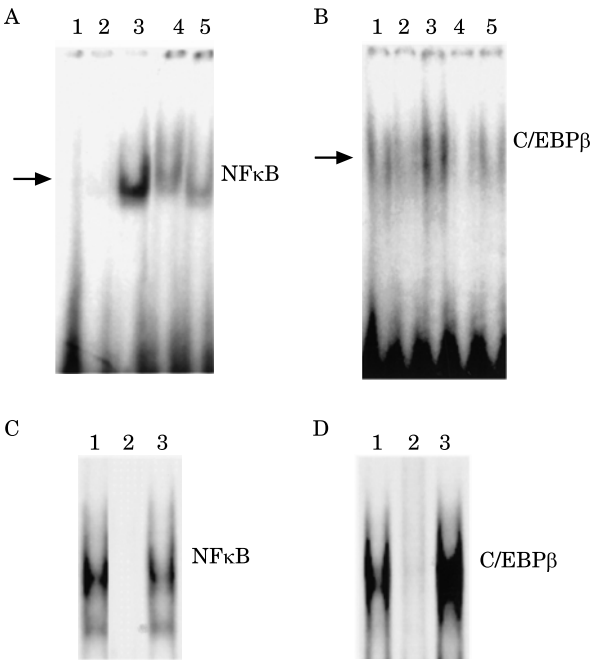

Figure 2. NFκB and C/EBPβ DNA binding activity following wounding.

Sub-confluent NHEK cultures were pre-incubated in KSM for 48 h. Monolayer cultures were wounded, the nuclear extracts collected and analyzed by EMSA. ^{32}P-labelled oligonucleotides containing the consensus binding sites for NFκB or C/EBPβ were used as probes. Lane 1, intact cells; lane 2, 15 min after wounding; lane 3, 30 min after wounding; lane 4, 60 min after wounding; lane 5, 120 min after wounding (C) NFκB and (D) C/EBPβ competitive binding. Lane 1, 30 min after wounding with vehicle; lane 2, 30 min after wounding plus 100-fold excess unlabeled oligonucleotide; lane 3, 30 min after wounding in the presence of 100-fold excess of AP-1 oligonucleotide. The open and closed arrows indicate the position of a free probe and the NFκB or C/EBPβ-probe complex, respectively.

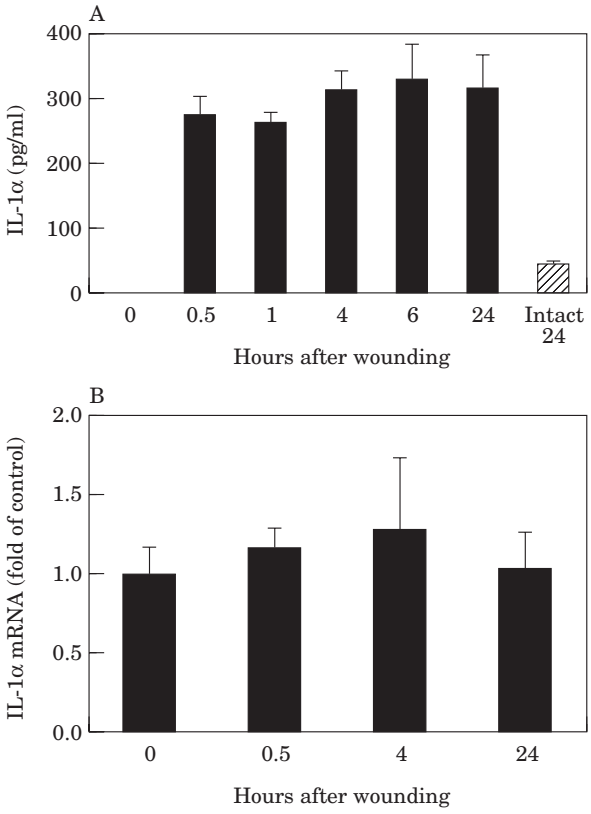

Figure 3. Time course for IL-1α protein release and IL-1α mRNA expression following wounding.

Sub-confluent NHEKs were pre-incubated in KSM without growth factors for 48 h. After the medium was changed to fresh KSM, monolayer cultures were wounded as described in Materials and Methods. (A) Culture media was collected after 0.5, 1, 4, 6 and 24 h after wounding, and assayed by ELISA. Non-wounded, intact cells incubated for 24 h in KSM served as control. (B) Total mRNA was collected after 0.5, 4 or 24 h after wounding, and RT-PCR performed. PCR products were electrophoresed on 2% (w/v) agarose gels and visualized by staining with ethidium bromide. Gels were scanned with a digital image analyzer and the data are expressed as fold-change compared to the intact control (0 h). The density of each amplified cDNA band for IL-1α was normalized relative to that of the corresponding band for G3PDH. Values represent mean ± SD of three replicate experiments.

IL-1α and wounding

The levels of released immunoreactive IL-1α from normal human epidermal keratinocytes (NHEKs) following wounding were quantified by enzyme-linked immunoabsorbent assay (ELISA) (Fig. 3A). While IL-1α was not detected in the supernatants of intact cell cultures at time 0, it appeared rapidly after wounding with over 250 pg/ml detected within 30 min. It is assumed that little, if any, additional IL-1α protein was released thereafter as indicated in the time curve. After 24 h in culture, supernatants from intact cultures contained approximately 30 pg/ml of IL-1α compared to 300 pg/ml in supernatants from wounded cultures. Wounding had no effect on IL-1α mRNA levels in NHEK cultures at any time point examined suggesting that the IL-1α detected represents preformed cytokine (Fig. 3B).

Activation of the NFκB and/or C/EBPβ transcription factors and subsequent expression of IL-6 is commonly mediated by proinflammatory cytokines such as IL-1 and TNF-α.[3] To determine whether IL-1α stimulated IL-6 secretion from NHEKs, cell cultures were incubated with recombinant human (rh) IL-1α, and IL-6 secretion was quantified by ELISA (Fig. 4A). A dose-dependent increase in IL-6 secretion was observed, with as little as 0.1 ng/ml of IL-1α inducing significant IL-6 production. IL-1α also induced the expression of IL-6 mRNA (data not shown).

To determine whether IL-1α or TNF-α are directly responsible for IL-6 expression in wounded keratinocytes, IL-1 receptor agonist (IL-1ra) or neutralizing antibodies to TNF-α were added to cell cultures prior to wounding and IL-6 mRNA expression was examined by quantitative (real-time) PCR. A marked increase in IL-6 expression occurred within 6 h

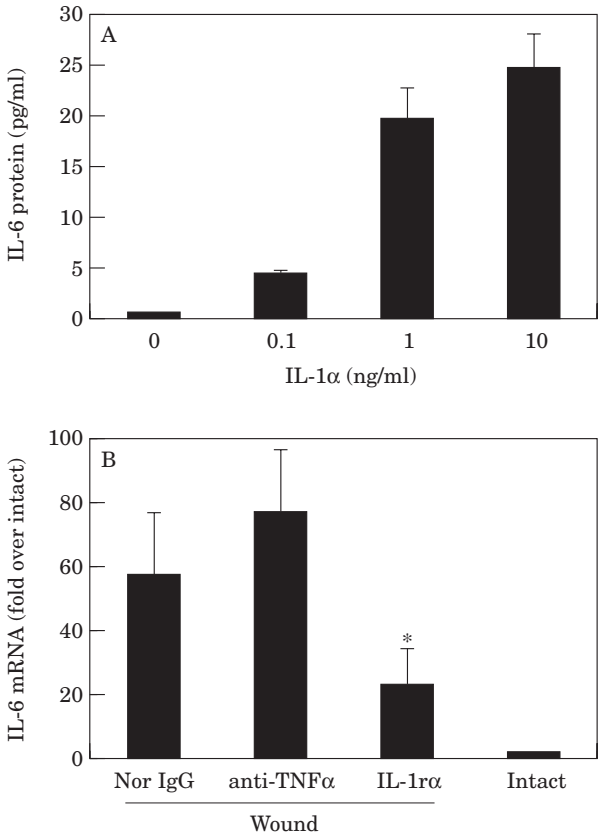

Figure 4. Influence of IL-1 on IL-6 expression.

(A) IL-6 secretion was determined in supernatants from NHEKs in response to IL-1α. Sub-confluent NHEK cultures were pre-incubated in KSM without growth factors for 48 h. The culture medium was changed to KSM containing rhIL-1α for an additional 6 h. IL-6 levels were determined by ELISA. Values represent mean ± SD of three replicate experiments. (B) Effect of IL-1 and TNF inhibitors on IL-6 mRNA expression in wounding. Sub-confluent NHEK cultures were pre-incubated in KSM without growth factors for 48 h. The medium was changed to KSM containing normal (nor) goat IgG (representing vehicle control), neutralizing antibodies to TNF-α or rhIL-1ra. The total mRNA were collected 1 h after wounding, and processed for quantitative real-time PCR. The measured value of each amplified cDNA for IL-6 was normalized relative to that of the corresponding value for G3PDH and the data are expressed as fold change compared to the intact control. Values represent mean ± SD of three separate experiments. Statistical significant: $*P<0.05$ vs control.

after wounding when compared to values from intact control cultures (Fig. 4B). IL-6 mRNA expression was significantly inhibited by IL-1ra, but not by neutralizing antibodies to TNF-α. The ability of cytokine inhibitors to prevent NFκB or C/EBPβ activation after wounding was also examined. Within 30 min following wounding, increased NFκB (Fig. 5A) and C/EBPβ (Fig. 5B) DNA binding was readily observed by EMSA. These activities were not affected when neutralizing antibodies to TNF-α were added (lane 3) but were inhibited by the addition of IL-1ra (lane 4).

Effects of IL-6 on keratinocyte proliferation and keratin expression

Studies were conducted to help determine the role IL-6 plays in wound healing and specifically re-epithelialization. Initially ^{3}[H]-thymidine (TdR) incorporation was measured in the presence or absence of IL-6 to determine whether IL-6 was mitogenic for keratinocytes (Fig. 6A). Although the results suggested a positive trend, there were no statistically significant mitogenic effects on keratinocytes by IL-6 after either 24 or 48 h of culture. Likewise, the addition of human recombinant IL-6 to NHEK cultures did not affect significantly cell growth, as determined by formazan dye formation (Fig. 6B).

K1/K10 and K16/K17 keratin mRNA expression was examined by RT-PCR in keratinocytes following IL-6 treatment or wounding (Fig. 7). The expression for keratins 1/10, which are expressed on the surface of differentiated keratinocytes,[19] was increased in intact cells following culture for 24 h and further increased in the presence of high (1.5 mM) calcium. The increase in expression of these keratins was inhibited by wounding as well as by the addition of exogenous IL-6 in the presence of calcium at either low or high concentrations. Consistent with the proliferation results, neither wounding nor the addition of exogenous IL-6 affected mRNA levels of keratins 16/17, which are found on proliferating keratinocytes.[20]

DISCUSSION

Wound healing is a complex process that involves an orderly sequence of events including coagulation, inflammation, formation of a provisional collagen matrix (granulation tissue), re-epithelialization and remodeling.[21] Originally, the central role for the inflammatory response in wound healing was believed to provide for an influx of inflammatory cells to the injured site to help stave off infection and remove cellular debris. Recently, we demonstrated a more direct role for inflammatory mediators, and particularly IL-6, in wound repair when delayed wound healing was found to occur in immunosuppressed or IL-6 deficient transgenic mice and was fully restored by administration of IL-6 protein or an IL-6 containing DNA plasmid.[5] Employing in situ hybridization, we further determined that during wound healing, IL-6 mRNA is expressed at high levels in epidermal keratinocytes located at the leading edge of the wound. Several lines of evidence support a role for IL-6 in wound healing. Low IL-6 activity was detected in wound fluid from diabetic mice, which normally display impaired wound healing.[22] Wound healing was also delayed in transgenic mice whose epidermal

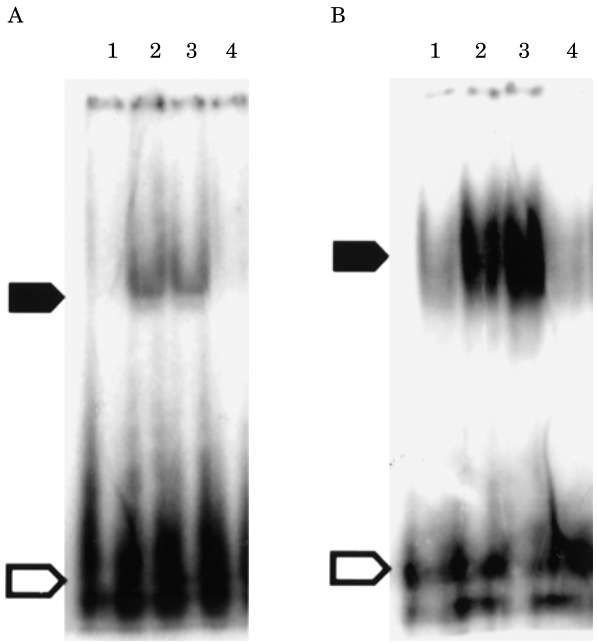

Figure 5. Effect of cytokine inhibitors on NFκB and C/EBPβ DNA binding activity following wounding.

Sub-confluent NHEK cultures were pre-incubated in KSM without growth factors for 48 h and the medium changed to KSM containing normal goat IgG, neutralizing antibodies to TNF-α or IL-1RA. The nuclear extracts were collected 30 min after wounding and analyzed by EMSA. ^{32}P-labelled oligonucleotides containing the consensus binding site for NFκB or C/EBPβ were used as probes. Lane 1, intact cells; lane 2, 30 min post-wounding; lane 3, wounded cells in the presence of TNF-α antibodies; lane 4, wounded cells in the presence of IL-1ra. The open and solid arrows indicate the position of the free and NFκB or C/EBPβ probe complexes respectively.

keratinocytes lack Stat3, a functional signal transducer activated by IL-6.[23,24]

IL-6 is a pleiotypic cytokine exerting multiple biological activities, most often associated with host defense mechanisms. Although its role in the skin is not well understood, it is produced at very low-levels in normal human epidermis, while abundant in many pathological conditions including psoriasis,[10] certain skin cancers such as Kaposi's sarcoma,[25] scleroderma,[26] allergic contact dermatitis[27] and thermal injury.[28] In these disease processes, IL-6 can be found in a variety of cell types including polymorphonuclear leukocytes, macrophages, fibroblasts, lymphocytes and endothelial cells in the dermis and epidermis.[9] In order to help study the mechanisms responsible for IL-6 production and its role in wound healing, it was useful to employ a defined model system. Thus, an in vitro wounding model[17,18] was selected which, while limited, since it does not provide an opportunity to study interactions between cell types in the healing process, provided an opportunity to study a reproducible response in keratinocytes. Employing this model, we demonstrated that de novo IL-6 is rapidly produced

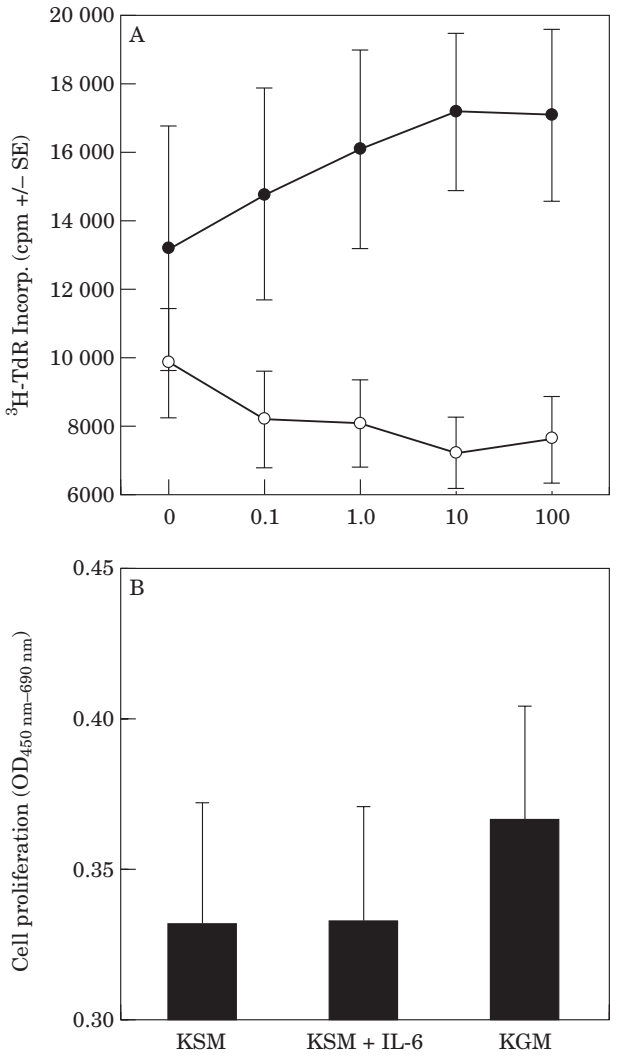

Figure 6. Effects of IL-6 on keratinocyte blastogenesis and proliferation.

(A) NHEK cultures were prepared as described in the Materials and Methods and pulsed with ^{3}H-TdR (1 μCi/well) for the last 4 h of incubation following 24 (open) or 48 h of culture (closed). (B) Cell proliferation was assayed with WST-1 dye. NHEKs were pre-incubated in KSM without growth factors for 48 h and then incubated in KSM alone, KSM plus 100 ng/ml IL-6 or KGM for 20 h. WST-1 reagent was added to each well and the formazan dye formed was quantitated as a live cell number using a multiwell spectrophotometer after 4 h. Values are expressed as $OD_{450–690}$ and represent the mean ± SD of three separate experiments.

after wounding, supporting earlier observations that epidermal keratinocytes at the wound edge are a major source of IL-6.[5] Furthermore, it was observed that IL-6 induction is highly dependent upon the release of preformed IL-1α following keratinocyte damage. We only observed the release of immunoreactive IL-1α into the culture medium immediately after wounding, while there was no evidence of IL-1α mRNA transcription at any time point examined after wounding. This is consistent with studies demonstrating that only preformed IL-1α is released from damaged keratino-

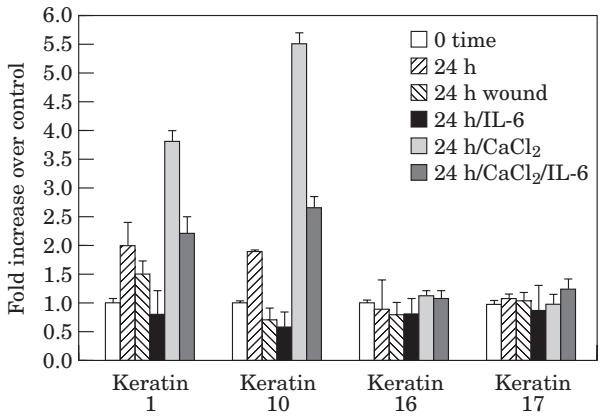

Figure 7. MRNA expression for keratins 1, 10, 16 and 17 in NHEK cultures.

NHEKs were pre-incubated in KSM without growth factors for 48 h and the medium replaced with fresh KSM. Cultures were wounded, incubated with 100 ng/ml IL-6 and/or cultured in the presence of high (1.5 mM) $CaCl_2$ concentrations. Open, time 0; slant-up, 24 h; slant-down, wounded; cross-lines, IL-6; horizontal lines, high Ca^{2+}; vertical lines, IL-6 plus high Ca^{2+}. Total RNA was collected at 0 or 24 h and subjected to RT-PCR. PCR products were electrophoresed on 2% (w/v) agarose gels and visualized by staining with ethidium bromide. Gels were scanned with a digital image analyzer and the data are expressed as fold-change compared to control. The density of each amplified cDNA band for keratin was normalized relative to that of the corresponding band for G3PDH. Values represent the mean ± SD of three separate experiments.

cytes.[29] Human keratinocytes constitutively produce biologically active pro-IL-1α and inactive pro-IL-1β[30,31] and this is released in response to exogenous stimuli such as UV-B exposure.[7] In wound healing, released IL-1α is postulated to be involved in fibroblast proliferation, collagenase secretion and synthesis of collagen, hyaluronic acid and glycosaminoglycan.[32]

Binding of NFκB and C/EBPβ transcription factors, to their respective cognate regulatory elements located in the promoter region of the IL-6 gene, is required for IL-6 expression.[6,33,34] Previous studies have shown that IL-1 can induce IL-6 transcription in the skin and this response is associated with the activation of NFκB[35] and, in this respect, Haas *et al.*[36] showed that NFκB is localized at the wound edge in cultured keratinocytes. Binding of IL-1α to the IL-1 type I receptor and IL-1 receptor associated proteins results in NFκB activation and phosphorylation of IκB.[37] In contrast to NFκB, C/EBPβ activity in the skin is regulated by mitogen-activated protein (MAP) kinases.[38] While various stimuli can activate C/EBPβ in the skin, IL-1 has yet to be examined. Here we demonstrate that both NFκB and C/EBPβ are activated by wounding with maximum activities occurring within 30 min, just prior to peak IL-6 mRNA expression. Furthermore, IL-1ra prevented activation of these transcription factors as well as IL-6 expression, whereas neutralizing antibodies to TNF-α had no effect. These results suggest that pre-formed IL-1α, released from wounded keratinocytes, is responsible for the induction of IL-6 and acts in an autocrine manner.

As mentioned previously, the ability of IL-6 to serve as a direct keratinocyte mitogen or growth factor is controversial as earlier studies demonstrating that IL-6 can directly stimulate proliferation of keratinocytes when grown in a basal medium[10] have been difficult to confirm in vivo.[11,12,39] Employing assays to measure both mitogenesis and proliferation, we were unable to demonstrate IL-6 involvement in keratinocyte replication. As a key contributor to both the structure and function of the skin, we also examined the expression of selected keratins following wounding, as changes occur in the types of keratin filaments expressed during re-epithelialization. For example, keratins K16 and K17 (type I) expression, while associated with chronic hyperproliferation, are found on keratinocytes near the wound edge following injury.[20,40,41] When mitotically active progenitor cells in the basal layer commit to terminal differentiation, a switch in expression from keratins K5/K14 to K1 (type II)/K10 (type I) occurs.[19] Differentiation of epidermal keratinocytes induced by culturing in high Ca^{2+} concentrations, increases the expression of keratins 1 and 10,[42] while growth factors, such as EGF and TGF-α, prevent differentiation and the expression of these keratins.[43,44] In the present study neither wounding, nor IL-6 effected K16/K17 keratin expression further suggesting that IL-6 does not directly influence keratinocyte growth. However, the expression of K1/K10 keratins was decreased as a result of either wounding or addition of IL-6. This was particularly evident when the cultures were treated with high concentrations of calcium suggesting that IL-6 influences differentiation processes.

In conclusion, these studies extend earlier in vivo investigations[5] demonstrating the importance of IL-6 in normal wound healing. Using an in vitro wound model, we demonstrate that de novo IL-6 is readily induced in keratinocytes following wounding through the release of preformed IL-1α which is responsible for the activation of keratinocytes involving NFκB and C/EBPβ transcription factors. The exact role of IL-6 in wound healing is currently unknown, but based upon our current understanding, the cytokine may have a limited role in proliferation, but may help regulate keratinocyte differentiation processes.

MATERIALS AND METHODS

Materials

Cryo-preserved NHEKs from breast skin of adult human females, keratinocyte basal medium (KBM) and KGM (KBM containing insulin, human epidermal growth factor, bovine pituitary extract, transferrin, hydrocortisone, gentamicin, and amphotericin-B), were obtained from

Clonetics Corp. (San Diego, CA, USA). Mouse anti-human TNF-α monoclonal antibody, rh=IL-1ra, normal goat IgG, rhIL-1α and rhIL-6 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). $CaCl_2$ was from Sigma Chemical Co. (St. Louis, MO, USA).

Keratinocyte monolayer culture

NHEK was cultured in KGM at 37°C in a humidified, 95% air/5% CO_2 atmosphere. Cells from their third passage were seeded into tissue culture plates at a density of 6×10^3–10×10^3 cells/cm^2 in KGM. When the cell density was approximately 70% confluent, each well or dish was washed twice with Hanks' balanced salt solution (HBSS) (Gibco, Rockville, MD, USA) and the medium was replaced with KBM containing transferrin, hydrocortisone, gentamicin and amphoterecin B (referred to as KSM). After incubation for 24 h, the cells were again washed twice with HBSS and cultured in fresh KSM for an additional 24 h or until they reached approximately 90% confluence.

Keratinocyte wounding model

The in vitro wounding model has been described.[17,18] Briefly, after washing twice with HBSS and changing to fresh KSM, NHEK cultures, at 90% confluency, were "wounded" by scraping with a tip of a 1 ml plastic pipette horizontally and vertically five, seven or 13 times across the bottom of 12-well, 6-well or 100 mm tissue culture plates, respectively. Intact cell cultures served as controls. In some experiments KSM containing 5 μg/ml of anti-TNF antibodies [neutralization dose $(ND)_{50}$=20–40 ng/ml], 100 ng/ml of IL-1ra (ND_{50}=7–10 ng/ml), 0.1–10 ng/ml of rhIL-α or, 1 μg/ml of normal IgG or 1.5 mM $CaCl_2$ was added prior to wounding.

RNA extraction and RT-PCR

Total cellular RNA was extracted from keratinocyte cultures using commercial reagents (Qiagen RNeasy kit; Valencia, CA, USA) according to manufacturer's instructions, and quantitated spectrophotometrically. Total RNA (1 μg) was reverse-transcribed into cDNA.[45] Five microlitres of each cDNA sample was amplified by RT-PCR using a GeneAmp PCR System 9600 DNA Thermal Cycler (PE Biosystems, Foster City, CA, USA) under conditions described.[45,46] PCR primers for human G3PDH, IL-6 and IL-1α were purchased from Clontech (Palo Alto, CA, USA). Primers for human keratins 1, 10, 16 and 17 were custom synthesized (Life Technologies, Bethesda, MD, USA) containing the folowing sequences:

Keratin 1:
5′ primer: 5′-TGACCCTGAGATCCAAAAGGTG-3′
3′ primer: 5′-CCGAATCCAACCGAGATTGAT-3′
Keratin 10:
5′ primer: 5′-ATGCAGAATCTGAATGACCGCT-3′
3′ primer: 5′-AAGTCATCAGCTGCCAGCCTT-3′
Keratin 16:
5′ primer: 5′-ACACATCCGTGGTGCTATCCA-3′
3′ primer: 5′-GGTTGGCACACTGCTTCTTGA-3′
Keratin 17:
5′ primer: 5′-GCTCAGCATGAAAGCATCCCT-3′
3′ primer: 5′-TTCCACAATGGTACGCACCTG-3′

The amplified PCR products were separated by electrophoresis on 2% (w/v) agarose gels (UltraPure, Sigma, St. Louis, MO, USA) at 75 V for 1 h and visualized by staining with 0.5 μg/ml of ethidium bromide. The gels were scanned with a digital imaging system (Eagle Eye II Image Analysis System, Stratagene, La Jolla, CA, USA), and the density of PCR bands was measured using Scion Image v3b (Scion Corp., Frederick, MD, USA). Measured values of IL-6 mRNA was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and expressed as fold-change from the mean value obtained from intact control cultures. G3PDH appears to be an appropriate housekeeping control under these conditions as it demonstrates similar differences in band intensity over a range of concentrations compared to 18 S ribosomal RNA (data not shown).

Real-time PCR was performed on selected samples in order to obtain quantitative values of mRNA. Reaction mixtures containing 5 μl of the cDNA sample, 12.5 μl of the master mix (PE Biosystems, Norwalk, CT, USA) and 1.25 μl of the primers and probe mixture for human G3PDH or human IL-6 (pre-developed TaqMan® assay reagents, PE Biosystems) were amplified according to the manufacturer's instructions using real-time PCR [TaqMan PCR System 5700 (PE Biosystems)]. Serial dilutions of control cDNA samples from NHEK stimulated with 10 ng/ml of phorbol myristate acetate (Sigma Chemical Co.) were measured simultaneously and used to generate a standard curve. The relative quantity of IL-6 mRNA was normalized with that of G3PDH and the results expressed as fold-change from mean values obtained from control cultures.

ELISA

Conditioned media from control and experimental NHEK cell cultures were collected, sterile-filtered, and stored at −70°C until assayed. Immunoreactive IL-6 and IL-1α was determined by commercial ELISA assays (R&D System Inc.) Results are expressed as the mean ± SEM for quadruplicate determination.

EMSA

To determine whether IL-6 associated transcription factors were activated by wounding, NFκB and C/EBPβ were analyzed by EMSA. Small-scale nuclear protein extracts were prepared from control and experimental keratinocyte cultures according to published methods.[47] DNA binding reactions and EMSAs were performed as described.[16] Briefly, 50 ng of oligonucleotides (Santa Cruz Biotechnology; Santa Cruz, CA, USA) were labeled with ^{32}P-ATP (NEN, Boston, MA, USA) using 4.5 U of phage T4 polynucleotide kinase (US Biochemical Corp., Amersham, Cleveland, OH, USA). Binding reactions were performed for 30 min at room temperature in reaction solutions (30 μl) containing 15 μl of binding buffer (40 mM HEPES, 32 mM KCl, 2 mM dithiolthreitol, 2 mM EDTA, 0.5 mg bovine serum albumin, 14% glycerol), 1 μg of poly(dI-dC), 3 μg of nuclear protein and 1 ng of ^{32}P-labeled probe. In some reactions a 50-fold excess of cold or mutated oligonucleotide was added to confirm specificity. For detection of DNA binding activity, the

following sequences of consensus or mutated oligonucleotide probes were used (binding sites are underlined):

NFκB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′
(mutant): 5′-AGTTGAGGCGACTTTCCCAGGC-3′
C/EBPβ: 5′-TGCAGATGCGCAATCTGCA-3′
(mutant): 5′-TGCAGAGACTAGTCTCTGCA-3′
AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′

Protein/DNA complexes were separated by electrophoresis on a 4% (w/v) polyacrylamide gel (Criterion™ Precast Gel, BioRad, Hercules, CA, USA) at 125 V buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA). The dried autoradiograms were scanned with a Storm 860 imaging system (Sunnyvale, CA, USA) and the results were examined using the One-Dscan gel analysis and the NIH Image 1.54 analysis software.

Cell mitogenesis and proliferation

NHEK in their third passage were seeded into 96-well culture plates at a density of 10^4 cells/cm^2 KGM and incubated for 24 h. The medium was changed to KSM and the cell cultures incubated for 48 h or until 30% confluent. The media was again replaced with either KSM or KSM containing rhIL-6. After 20 or 44 h, 1 μCi per well of ^{3}[H]-TdR was added (specific activity 6.7 Ci/mmol: Dupont NEN, Boston, MA, USA) and cultures incubated for an additional 4 h. The cells were detached from the plates with trypsin/EDTA solution and collected onto glass-fiber filters using an automated cell harvester (Skatron, Sterling, VA, USA). Radiolabelled incorporation of DNA was quantified by liquid scintillation counting. To measure cell proliferation, NHEK in their third passage were plated into 96-well tissue culture plates at a density of 10^4 cells/cm^2 in KGM and incubated for 24 h. After replacing the medium with KSM, cells were incubated for 48 h or until approximately 30% confluent. Cells were incubated again in KSM alone, KSM containing 100 ng/ml of IL-6 or KGM. After 20 h, cell proliferation was assessed using a commercial reagent (WST-1, Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions which measures the formation of formazan dye using an ELISA plate reader ($OD_{450–690}$).

Statistical analysis

All experiments were replicated and representative findings are shown. Statistically significant differences were determined by one-way ANOVA. When the F value was significant, the differences in mean values were evaluated by Dunnett's test. In all comparisons a value of $P<0.05$ was used to indicate significant differences.

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