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Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors

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Abstract

Although numerous epidemiological studies have shown that inorganic arsenicals are human skin carcinogens, there is currently no accepted mechanism for its action or an established animal model for its study. We observed increased mRNA transcripts and secretion of keratinocyte growth factors, including granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor-α (TGF-α) and the proinflammatory cytokine tumor necrosis factor-α (TNF-α) in primary human epidermal keratinocytes cultured in the presence of low micromolar concentrations of sodium arsenite. Total cell numbers, as well as c-*myc* expression and incorporation of [^{3}H]thymidine, both indicators of cell proliferation, were also elevated in keratinocyte cultures treated with sodium arsenite. As an in vivo model, the influence of arsenic on mouse skin tumor development was studied in transgenic TG.AC mice which carry the v-Ha-*ras* oncogene, and can serve as a genetically initiated model for skin carcinogenesis. Following low-dose application of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a marked increase in the number of skin papillomas occurred in transgenic mice receiving arsenic in the drinking water as compared to control drinking water. Papillomas did not develop in arsenic-treated transgenic mice that had not received TPA or arsenic-treated wild-type FVB/N mice, suggesting that arsenic is neither a tumor initiator or promoter but rather an enhancer. Injection of anti-GM-CSF antibodies following application of TPA in transgenic mice reduced the number of papillomas. Consistent with that observed in human keratinocyte cultures, increases in GM-CSF and TGF-α mRNA transcripts were found within the epidermis of arsenic-treated mice when compared to controls within 6 weeks of

Abbreviations: GM-CSF, granulocyte macrophage-colony stimulating factor; IL, Interleukin; NHEK, normal human epidermal keratinocytes; RT, reverse transcriptase; PCR, polymerase chain reaction; TGF, transforming growth factor; TNF, tumor necrosis factor

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treatment. These results suggest that arsenic enhances papilloma development via the chronic stimulation of keratinocyte-derived growth factors and represents the first example of a chemical carcinogen that acts in this manner. These studies suggest that in vitro studies with human keratinocyte cultures examined in conjunction with TG.AC transgenic mice can provide a useful model for examining the tumor enhancing properties of environmental chemicals.

Keywords: Arsenic carcinogenesis; Skin cancer, chemicals; Growth factor; Cytokine

1. Introduction

Chronic exposure to inorganic arsenicals through contaminated drinking water, medicinal agents or occupational exposure is associated with neoplasias of the skin and to a lesser extent, lung, kidney, bladder and liver [1,2]. Less severe dermatological effects, including hyperpigmentation and hyperkeratosis, appear on the palms and soles following arsenic exposure [3]. Epidemiological studies in humans have suggested that the population cancer risk due to arsenic in US water supplies may be comparable to that of environmental tobacco smoke and radon in homes with risk estimates of around 1 per 1000 [4]. In contrast to other chemical carcinogens in humans, the mechanisms involved in tumor formation by arsenic are not well established, due in part to lack of predictive animal models. Arsenic fails to promote neoplastic disease in either single-stage or two-stage murine models [5,6] or to induce mutations in bacteria or Chinese hamster cells [7,8]. However, arsenic is co-mutagenic with ultraviolet radiation, X-rays or alkylating agents and induces SCEs in human lymphocytes [9]. Arsenic also induces gene amplification in mouse 3T3 cells and, thus, has been suggested to serve as a tumor progressor rather than an initiator or promoter [10].

In response to environmental stimuli, keratinocytes can produce and secrete a number of inflammatory and chemotactic cytokines, such as interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and IL-8, as well as cellular growth factors including transforming growth factor-α (TGF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF) which have been implicated in various pathological processes such as contact hypersensitivity, psoriasis and neoplasia [11–13]. In particular, overexpression of TGF-α has been associated with the pathological process of neoplastic transformation in the skin and is readily secreted in cultured cells following transformation with viral and cellular oncogenes or by treatment with tumor promoters [11,14]. When injected into initiated mouse skin, TGF-α induces DNA synthesis in epidermal cells [15] and keratinocytes transfected with a constitutive TGF-α transgene develop benign skin papillomas when grafted to nude mice [16]. Using transgenic mice, it was shown recently that targeted overexpression of TGF-α to the epidermis elicits hyperplasia, hyperkeratosis and spontaneous squamous cell carcinomas [17,18]. Furthermore, TGF-α transgenic mice exhibit keratinocyte hyperproliferation and neoplasias in the pancreas, liver and mammary epithelia [19–21], as well as an accelerated response to chemical-induced cancers [22], suggesting that TGF-α overexpression has the unique ability to complement both initiation and promotion by serving to enhance the growth of neoplastic cells. Based upon the aforementioned observations, we hypothesized that arsenic may induce inflammation and/or tumorigenesis by modulation of keratinocyte-derived inflammatory or growth promoting cytokines, respectively.

2. Materials and methods

2.1. Cell cultures

Cryopreserved, normal human epidermal keratinocytes (NHEK) from breast skin of adult females were purchased from the Clonetics Corp. (San Diego, CA). NHEK were grown at a low calcium concentration (150 μM) at 37°C, 5% CO_2 in keratinocyte basal medium supplemented with 5 μg/ml insulin, 0.1 ng/ml recombinant epidermal growth factor, 0.4% bovine pituitary extract, 0.5 μg/ml hydrocortisone, 50 μg gentamicin/ml and 50 ng amphotericin-B/ml (henceforth referred to as keratinocyte growth medium or KGM; Clonetics). NHEK in passages 1–3 were subcultured at seeding densities of

2500–20000 cells/cm^2 in KGM. When the cells were 55–65% confluent, the medium was changed to KGM without hydrocortisone and bovine pituitary extract (a source of TGF-α). The cells were allowed to grow for an additional 24 h and fresh KGM without hydrocortisone and bovine pituitary extract was added in the presence of sodium arsenite (Sigma Chemical Co., St. Louis, MO). The cultures were allowed to incubate for an additional 2 to 18 h and the cells were collected for RNA isolation or the supernatants collected for cytokine quantitation. Cell viability was determined by trypan blue exclusion and confirmed by quantitating aliquots of the supernatants for the presence of lactate dehydrogenase (LDH; 23).

2.2. Cell proliferation

Cell proliferation was measured by quantitating cell growth after seeding in 25-cm^2 tissue culture flasks. Keratinocyte cultures were allowed to establish for 24 h, after which time triplicate cultures were treated with sodium arsenite and allowed to grow to approximately 75% confluency in KGM. The cells were removed from the flasks by trypsinization for 2 min with 0.025% trypsin/0.01% EDTA in HBSS (Clonetics), washed 1 × in HBSS and counted in a hemocytometer. A second indicator of cell proliferation, incorporation of [^{3}H]thymidine into cellular DNA, was also examined. Briefly, 1.5×10^3 cells/well were cultured in 96-well U-bottom culture plates (Costar, Cambridge, MA) for 48 h in KGM and then treated with sodium arsenite. After 24 h the cells were pulsed with 1 μCi per well of [^{3}H]thymidine (specific activity 6.7 Ci/mmol, Dupont NEN, Boston, MA) and incubated for an additional 48 h. The cells were detached from the plates with trypsin/EDTA solution (Clonetics) and collected onto glass-fiber filters using an automated cell harvester (Skatron, Sterling, VA). [^{3}H]Thymidine incorporation was quantitated by liquid scintillation counting.

2.3. Cytokine secretion

GM-CSF and IL-1α concentrations were determined by ELISA using commercially available systems (Genzyme, Cambridge, MA) and TNF concentrations measured by a cytolytic assay using L929 fibroblast cells treated with actinomycin D [24]. Results are expressed as the mean for quadruplicate determinations from one of three representative experiments. Immunoreactive TGF-α was determined by Western blotting from supernatants following concentration using a centrifugal concentrator (Centricon 10; Amicon, Danvers, MA). The samples were boiled in Laemmli sample buffer for 5 min and then applied to a 10–20% SDS–polyacrylamide gel. After electrophoresis, the gel was equilibrated for 30 min in 20 mM Tris, 150 mM glycine, 20% methanol, pH 8.8 (Tris buffer) and transferred overnight to a nitrocellulose membrane at 100 mA. TGF-α was visualized by using a 1:100 dilution of polyclonal goat anti-human TGF-α antibody (R&D Systems, Minneapolis, MN) and rabbit anti-goat Ig conjugated to alkaline phosphatase as the secondary antibody (Organon Teknika, West Chester, PA). The membranes were scanned with a computerized laser densitometer (LKB).

2.4. mRNA Extraction and reverse transcriptase–polymerase chain reaction (RT–PCR)

Cultured NHEKs or mouse skin samples (shaved dorsal area) were collected, homogenized in a small volume of RNAzol B^{TM} solution (Biotecx Laboratories, Houston, TX), and total cellular RNA extracted according to the manufacturers instructions. RNA was dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the poly(A$^+$) mRNA fraction isolated by binding to oligo d(T) cellulose spin columns (Invitrogen Corp., San Diego, CA). For the synthesis of cDNA, 0.1 μg of mRNA from each sample was resuspended in a 20 μl final volume of the reaction buffer (25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP (Perkin Elmer, Cetus, Norwalk, CT)) and 0.5 μg oligo d(T) 12–18 primer (BRL, Gaithersburg, MD). After the reaction mixture reached 42°C, 400 U SuperScript^{TM} reverse transcriptase (BRL) (200 U/μl) was added to each tube and incubated for 30 min at 42°C. The reaction mixture was stopped by denaturing the enzyme at 99°C for 5 min and was diluted with distilled water to a volume of 50 μl. Commercially available PCR primers for human TGF-α, IL-1α, GM-CSF, c-*myc*,

β-actin and G3PDH, as well as mouse GM-CSF, TGF-α, IL-6 and G3PDH were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The primers contained the following sequences:

Human TGF-α 5′ ATGGTCCCCTCGGCTGGACAG 3′ 5′ GGCCTGCTTCTTGTGGCTGGCA 3′; amplified PCR fragment: 297 bp

Human IL-1α 5′CAAGGAGAGCATGGTGGTAGTAGCAACCAACG 3′ 5′ TAGTGCCGTGAGTTTCCCAGAAGAAGAGGAGG 3′; amplified PCR fragment: 491 bp

Human TNF-α 5′ GAGTGACAAGCCTGTAGCCCATGTTGTAGCA 3′ 5′ GCAATGATCCCAAAGTAGACCTGCCCAGAC 3′; amplified PCR fragment: 444 bp

Human GM-CSF 5′ ATGTGGCTGCAGAGCCTGCTGC 3′ 5′ CTGGCTCCCAGCAGTCAAAGGG 3′; amplified PCR fragment: 424 bp

Human c-*myc* 5′ TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT 3′ 3′ TCTTGACATTCTCCTCGGTGTCCGAGGACCT 3′; amplified PCR fragment: 478 bp

Human β-actin 5′ ATGGATGATGATATCGCCGCG 3′ 5′ CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC 3′; amplified PCR fragment: 838 bp

Human G3PDH 5′ TGAAGGTCGGAGTCAACGGATTTGGT 3′ 5′ CATGTGGGCCATGAGGTCCACCAC 3′; amplified PCR fragment: 983 bp

Mouse GM-CSF 5′ TGTGGTCTACAGCCTCTTCAGCAC 3′ 5′ CAAAGGGGATATCAGTCAGAAAGGT 3′; amplified PCR fragment: 368 bp

Mouse IL-6 5′-ATGAAGTTCCTCTGCAAGAGACT 3′ 5′ CACTAGGTTTGCCGAGTAGATCTC 3′; amplified PCR fragment: 638 bp

Mouse TGF-α 5′ GGACAGCTCGCTCTGCTAGCG 3′ 5′ CTTCTCGTGTCTGCAGACGAG 3′; amplified PCR fragment: 410 bp

Mouse G3PDH 5′-TGAAGGTCGGTGTGAACGGATTTGGC 3′ 5′ CATGTAGGCCATGAGGTCCACCAC 3′; amplified PCR fragment: 983 bp

Five-μl aliquots of the synthesized cDNA (corresponding to 10 ng of mRNA) were added to 45 μl of PCR mix containing 5 μl of 10× PCR buffer, 1 μl deoxynucleotides (1 mM each), 0.5 μl of sense and anti-sense primers (0.15 μM) and 0.25 μl DNA polymerase (GeneAmp™ PCR, Perkin Elmer Cetus, Norwalk, CT). The reaction mixture was covered with Ampli (Gem) wax tablet (Perkin Elmer Cetus). Amplification was initiated by 1 min of denaturation at 94°C for 1 cycle followed by 25, 30 or 35 cycles at 94° for 30 s, 55°C for 30 s and 55°C for 1 min using GeneAmp PCR System 9600 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, the samples were incubated for 7 min at 72°C. RNA concentrations and PCR cycles were titrated to establish standard curves, to document linearity and to permit semi-quantitative analysis of signal strength as previously described [25]. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 33 and 35 cycles to define optimal conditions. When appropriate, the specificity of the PCR bands was confirmed by restriction enzyme analysis of the amplified cDNA which generated restriction fragments of the expected size (data not shown).

The PCR products were visualized by UV illumination following electrophoresis through 2.0% agarose (UltraPure, Sigma) at 60 V for 80 min and staining in Tris Borate-EDTA buffer (89 mM Tris, 89 mM Boric acid, 2.5 mM EDTA, pH 8.2) containing 0.5 μg/ml ethidium bromide. Gels were photographed with type 55 positive/negative film (Polaroid; Cambridge, MA). The films were scanned with a computerized laser densitometer and the area under the curve normalized for G3PDH content.

2.5. Animals

Female, homozygous TG.AC mice, containing a v-Ha-*ras* structural gene (with mutations at codons 12 and 59) fused to a fetal γ-globin promoter and linked to a simian virus 40 polyadenylation/splice sequence [29], and nontransgenic FVB/N mice were obtained from Taconic Farms (Germantown, NY). Mice were maintained in our animal facility at least 1 week before use in compliance with NIEHS approved guidelines for the humane treatment of laboratory animals. Mice were fed Purina Pico Chow No. 5058 and water ad libitum and kept on a 12-h light/12-h dark cycle. Groups of the wild-type FVB/N or TG.AC transgenic mice were provided 0.02% arsenic (as sodium arsenite, Sigma Chemical Co.) in their drinking water. Four weeks later, the

dorsal skin was shaved with electric clippers and 24 h later an initial dose of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) was applied topically in 200 μl of acetone. Animals were shaved thereafter during TPA dosing on an as needed basis. Mice received 2.5 μg TPA twice per week for 2 weeks. Selected groups of arsenic-treated transgenic mice were administered intravenous injections of 50 μg of a monoclonal antibody to mouse GM-CSF (Genzyme, Cambridge, MA) in 0.2 ml PBS, 2 h following each application of TPA. Papilloma incidences were recorded three times per week for 14 weeks. Animals were euthanized using CO_2 narcosis and skin samples collected.

2.6. Histology

Samples of dorsal skin from control and arsenic-treated mice were preserved in 10% buffered formaldehyde. Paraffin-embedded tissue sections (5–6 mM thickness) were prepared and stained with hematoxylin and cosin.

2.7. Statistical analysis

Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 Multicomparison procedure using Wilkes–Shapiro test for normality and Dunnett's test for multiple comparisons with a common control group. When variances were nonhomogeneous, multiple comparisons utilizing the Bonferroni adjustment of the Student *t*-test were performed. Statistically significant differences were reported when the *p*-value was less than 0.05.

3. Results and discussion

Initially, we determined whether arsenic alters cytokine secretion in normal human epidermal keratinocytes. Cell viability, as assessed by trypan blue dye exclusion, indicated that within the time period examined only arsenite concentrations above 8 μM affected cell viability (< 12% decrease at 8 μM; Fig. 1A). At non-cytotoxic concentrations, sodium arsenite induced a dose-dependent increase in secreted GM-CSF and TNF-α (Fig. 1B) as well as immunoreactive TGF-α (Fig. 1C, D) within 18 h following addition to the keratinocyte cultures. Neither proinflammatory cytokines, such as IL-1α (Fig. 1B) or IL-6 (data not shown), nor chemokines, including IL-8 or MCP-1 (data not shown), were secreted in response to arsenic. The cytokine pattern produced by arsenic was unlike earlier studies where we showed that contact irritants and contact sensitizers induced proinflammatory cytokines and chemokines rather than growth factors [23]. Although GM-CSF can participate in inflammatory reactions, it also causes keratinocyte proliferation [26,27] and has been implicated in tumor promotion via mediating inflammatory cell influx and increasing dark cell numbers in mouse skin [13]. GM-CSF is also actively transcribed during the tumor promotion process being involved in leukocyte migration and activity [27].

To determine whether the increased concentrations of growth factors in culture supernatants were due to new transcription or release of preformed protein, poly(A^+) mRNA isolated from arsenic-treated keratinocytes was examined by RT–PCR. The constitutively expressed genes, G3PDH and β-actin were used to adjust for equal mRNA concentrations. As shown in Fig. 2, within 4 h following addition of arsenic at concentrations ranging from 0.5 to 4 μM to the keratinocyte cultures, increases in TNF-α, GM-CSF and TGF-α mRNA transcripts were evident. In some instances addition of 10 nM TPA, was used as a positive control. Consistent with the increased growth factor response, c-*myc* protooncogene expression was also increased. c-*myc* is associated with keratinocyte cell proliferation [28], and its expression suggests that growth factor secretion induced by arsenic was accompanied by increased cell proliferation. To confirm this, cell growth was examined in keratinocyte cultures treated with sodium arsenite. Low levels of sodium arsenite (0.001–0.005 μM) significantly stimulated keratinocyte proliferation (Fig. 3), while levels of arsenic associated with cytotoxicity, as expected, decreased cell numbers and [^{3}H]thymidine incorporation (data not shown).

Based upon the aforementioned observations, we hypothesized that arsenic may enhance tumorigenesis by modulation of keratinocyte-derived growth promoting cytokines, such as GM-CSF and TGF-α

and, thus, serve as a tumor enhancer. To address this question, a model was developed using the transgenic mouse strain TG.AC. The transgene carries a v-Ha-*ras* oncogene fused to a zeta globin promoter [29] which confers the properties of a genetically initiated skin. Wounding, or topical application of TPA or other tumor promoters to the shaved dorsal skin readily induces squamous cell papillomas which can progress to squamous cell carcinomas or sarcomas [30,31]. By applying limited doses of TPA (2.5 μg; 2 times/week; 2 weeks) to the dorsal side of shaved skin of TG.AC mice, a modest incidence of papillomas (mean of 3 per mouse) develop within 4 weeks (Spalding, unpublished observation). Wild-type FVB/N or TG.AC transgenic mice were provided 0.02% arsenic (as sodium arsenite) in the drinking water, and 4 weeks later groups of mice were subjected to this TPA regimen. While no papillomas were observed in animals receiving control water or sodium arsenite only, a number of skin samples from arsenic-treated animals showed epidermal thickening and hyperkeratosis (Fig. 4A, B), consistent with that observed in humans after arsenic exposure [3]. As shown in Fig. 5A, within 6 weeks following TPA application, the incidence of papillomas in arsenic-exposed, TPA-treated TG.AC mice was approximately 4-fold higher than in promoted mice that had not received arsenic suggesting that arsenic can serve to enhance papilloma formation. The incidence decreased to 2-fold 10 weeks following TPA-treatment most of which was due to the death of animals with heavy papilloma burdens (not shown). Papillomas were not observed in non-promoted TG.AC mice (Fig. 5A) or TPA-promoted, wild-type FVB/N mice that had received arsenic when observed up to 3 months (data not shown), indicating that arsenic does not act as a tumor promoter or initiator, respectively. Since GM-CSF was expressed in keratinocyte cultures following addition of arsenic and has been implicated in skin neoplasia

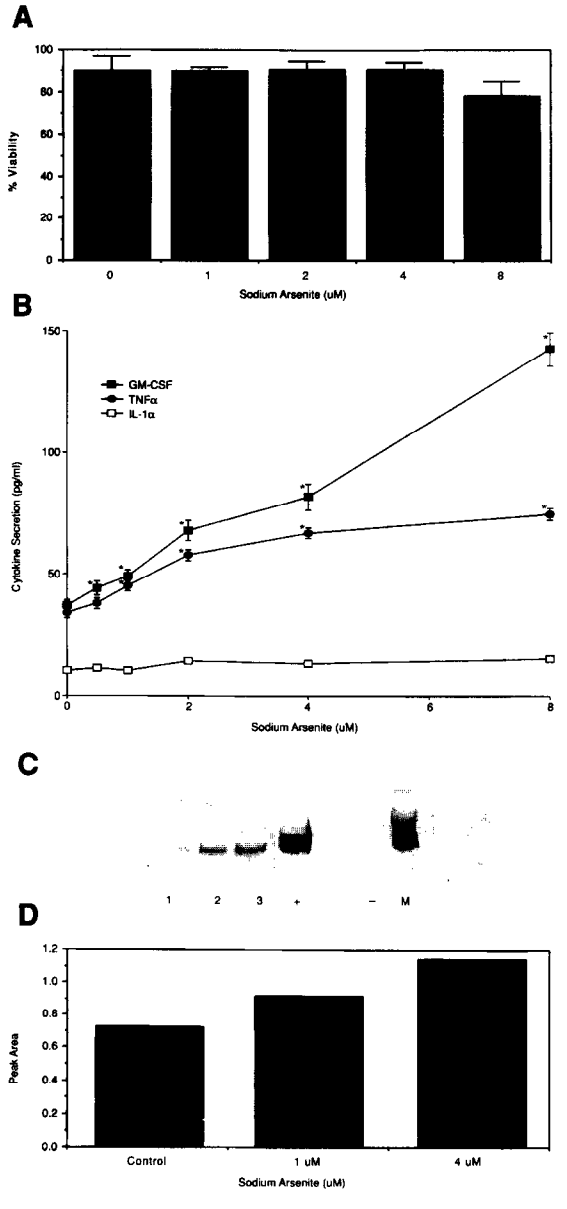

Fig. 1. Viability and cytokine secretion from normal human epidermal keratinocytes grown in the presence of arsenite for 18 h. (A) Cell viability as determined by Trypan blue exclusion. (B) GM-CSF and IL-1α concentrations were determined by ELISA using commercially available systems (Genzyme, Cambridge, MA) and TNF-α concentrations measured by a cytolytic assay using L929 fibroblast cells treated with actinomycin D. Results are expressed as the mean for quadruplicate determinations from one of three representative experiments. (C) Immunoreactive TGF-α as determined from culture supernatants by immunoblotting. TGF-α was visualized by using a polyclonal goat anti-human antibody diluted 1:100 and rabbit anti-immunoglobulin conjugated with alkaline phosphatase as the secondary antibody. Lane 1, 0 μM; lane 2, 1 μM; lane 3, 4 μM; +, TGF-α std; −, water control; M, 30 kDa molecular weight standard. (D) Computerized laser densitometer scan of C.

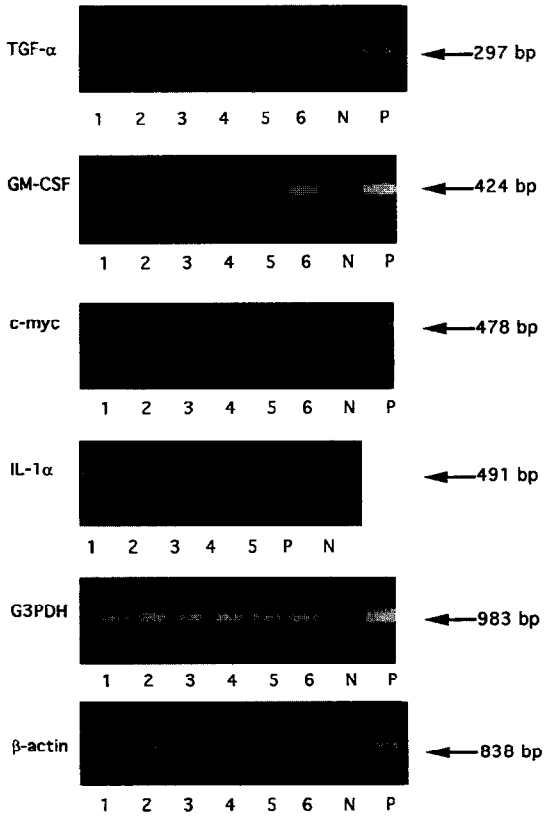

Fig. 2. Amplification of TGF-α, GM-CSF, TNF-α, *c-myc*, IL-1α, G3PDH and β-actin mRNAs by RT–PCR. Cells were treated as described in Fig. 1 except that cultures were terminated after 4 h exposure to sodium arsenite or 10 nM TPA. RNA concentrations and PCR cycles were titrated to establish standard curves, to document linearity and permit semi-quantitative analysis of signal strength. Lane 1, 0 μM; lane 2, 0.5 μM; lane 3, 1.0 μM; lane 4, 2.0 μM; lane 5, 4.0 μM arsenite; lane 6, 10 nM TPA; N, water control; P, commercial positive control.

[13,26,27], we examined whether neutralization of GM-CSF in vivo affected papilloma formation. As shown in Fig. 5B, administration of monoclonal antibodies to GM-CSF following application of TPA significantly reduced the average number of papillomas over the entire time period examined. Histologically, papillomas were similar to those reported previously in TG.AC mice promoted with TPA [30,31] and were predominantly squamous cell with some basal cell papillomas that originated in hair follicles (Fig. 4C). Consistent with these observations, two cell types in arsenic-induced skin tumors have been identified in humans; basal cell carcinomas and squamous cell carcinomas arising in keratotic areas [1,32]. The basal cell carcinomas are usually only locally invasive while squamous cell carcinomas may have distinct metastases.

TGF-α, and to a slight extent GM-CSF, are expressed constitutively in normal skin although the latter is not found in unstimulated cultured keratinocytes [12,13,23]. To determine whether arsenic exposure modulates growth factor expression in vivo,

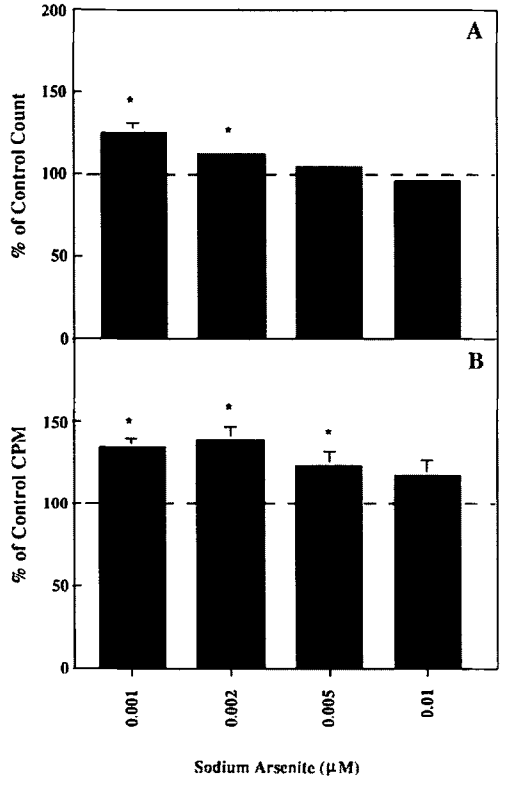

Fig. 3. Stimulation of cell proliferation in keratinocytes by sodium arsenite. Keratinocytes cultures were allowed to establish in 25-cm^2 culture flasks or 96-well U-bottom culture plates as described in the Section 2 and then exposed to sodium arsenite. (A) Triplicate cultures of 25-cm^2 flasks were trypsinized and counted to quantitate cell numbers. Cell viability was determined by trypan blue exclusion. Each bar represents the mean percent of viable control cell counts ± SE. (B) Twelve wells were used for each arsenite concentration for thymidine incorporation studies. The cells were harvested and incorporated radiolabel quantitated as described in Section 2. Each bar represents the mean percent of control CPM ± SE from three replicate experiments. * Significantly different from control cultures at $p < 0.05$.

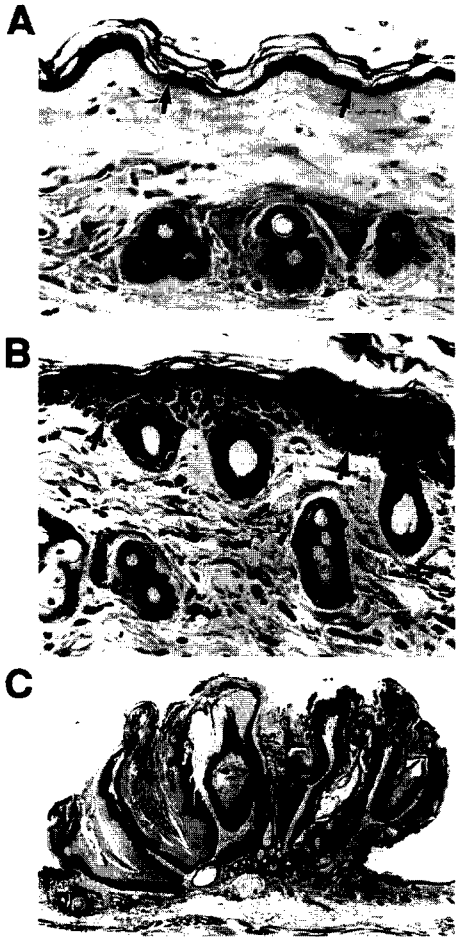

Fig. 4. Histology of murine skin after arsenic treatment. TG.AC transgenic mice were given 0.02% sodium arsenite in the drinking water starting at 12 weeks of age. H&E, 340×. (A) Normal skin. The epithelium (arrows) is thin with a moderate amount of keratin on the surface of the skin. (B) Mouse skin after sodium arsenite only. The epithelium (arrows) is minimally thickened (acanthosis) with a moderate amount of stratum corneum on the surface of the skin. (C) Squamous cell papilloma from an arsenic-treated mouse promoted with TPA.

epidermal samples were collected from non-promoted control and arsenic-treated mice and mRNA transcripts for relevant cytokines were examined by RT–PCR (Fig. 6). GM-CSF and TGF-α mRNA transcripts were highly expressed in randomly selected, arsenic-treated mice compared to mice receiving only water while no differences in the expression of the inflammatory cytokine, IL-6, was observed between the two groups. TGF-α and GM-CSF immunostaining was also markedly increased in randomly selected skin sections from TG.AC mice that had received arsenic in their drinking water (data not shown).

Arsenic, an ubiquitous element, represents a human health concern when concentrated in the environment from natural or anthropogenic processes. Epidemiological studies have shown a significant dose-response relationship between chronic exposure to inorganic arsenic in drinking water and mortality from skin and lung cancers [1,2,33]. In particular, skin cancers, including Bowen's disease, basal cell carcinoma and squamous cell carcinoma have been observed in patients treated with Fowler's solution,

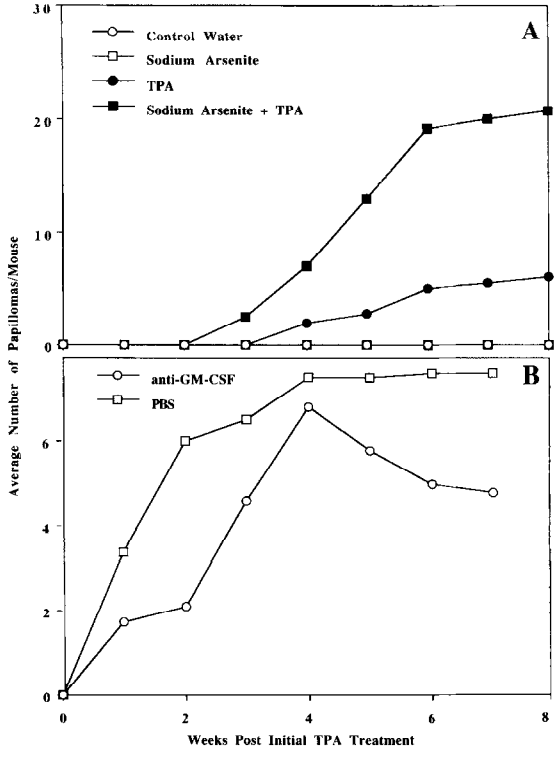

Fig. 5. Papilloma incidences in TG.AC transgenic mice given 0.02% sodium arsenite in the drinking water starting at 12 weeks of age. (A) Mice were pretreated with arsenite or water for 4 weeks and sub-groups were treated four times with 2.5 μg of TPA in acetone (administered twice per week for two consecutive weeks). Papillomas were not observed in non-TPA-promoted, arsenic-treated transgenic mice or wild-type FVB/N mice (not shown). $n = 20$ per group. (B) Treated as above except mice were administered intravenously monoclonal antibodies to mouse GM-CSF or PBS 2 h following each application of TPA ($n = 6$).

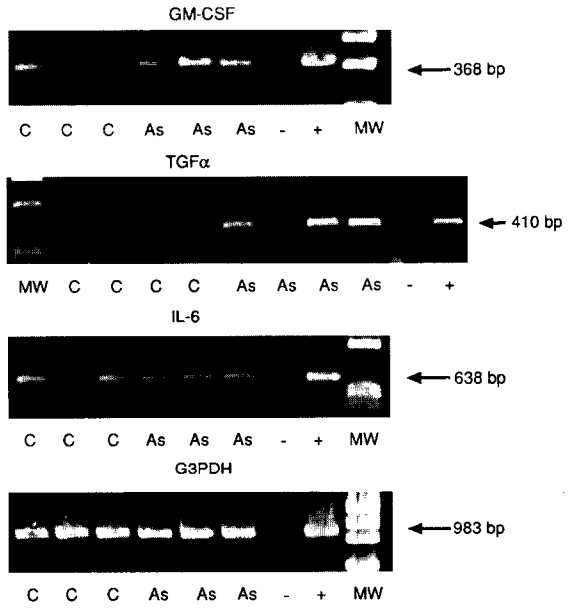

Fig. 6. GM-CSF and TGF-α expression in skin of TG.AC transgenic mice. Poly(A^+) mRNA was isolated from skin samples taken from the back of TG.AC transgenic mice treated with control or arsenite (0.02%) in the drinking water for 6 weeks. RT–PCR was used to determine relative differences in GM-CSF, TGF-α and IL-6 transcripts as described in Section 2. −, water control; +, commercial positive control; MW, molecular weight markers.

arsenic-exposed pesticide workers and residents of areas where arsenic-contaminated drinking water has been found [1,2,32,33]. The prevalence of skin cancer in humans is high, ranging between 5% and 10% following ingestion of 1.5–6.0 g of arsenic over a short period or 20 g over a lifetime [33]. These exposure levels are approximately 5-fold lower than the concentrations used in our in vivo studies. Considering the accumulating evidence that keratinocyte growth factors enhance skin tumor formation, the present data suggest that arsenic may exert its carcinogenicity through chronic, low-level stimulation of keratinocyte-derived growth factors rather than genetic events. In this respect, arsenic was recently shown to inhibit keratinocyte differentiation, as evidenced by inhibition of involucrin, a cell differentiation marker [34]. The high affinity of arsenic for sulfhydryl groups leads to its accumulation and tenacious retention in keratin-rich tissues such as hair and skin and tissue levels of arsenic have been used as a quantitative indicator of exposure in humans [35]. Analysis of hair samples collected from mice in the present studies showed that the arsenic content reached 329 ng/g within 4 weeks following exposure compared to 0.6 ng/g for controls. This analysis, along with the immunohistological studies localize both the chemical and the overexpression of growth promoting cytokines to the hair follicles. Consistent with these observations, most skin papillomas originate in hair follicles where the v-Ha-*ras* transgene is also expressed in TG.AC mice [36], thus providing a supportive environment for tumor development. In addition to providing a mechanistic-based hypothesis for arsenic carcinogenicity, the present findings indicate that cultured human epidermal keratinocytes and the TG.AC transgenic mice can be useful models for assessing the tumor-enhancing properties of chemical carcinogens.

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