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Arsenic Induces Overexpression of Growth Factors in Human Keratinocytes

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Although epidemiological studies have shown that inorganic arsenicals are human skin carcinogens and induce hyperproliferation and hyperkeratosis, there is currently no known mechanism for their 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 (TGFα) and the proinflammatory cytokine tumor necrosis factor-α in primary human epidermal keratinocytes cultured in the presence of low micromolar concentrations of sodium arsenite. Treatment with sodium arsenite resulted in a significant increase in cell proliferation, as indicated by increases in cell numbers, *c-myc* gene expression, and incorporation of [^{3}H]thymidine into cellular DNA. Studies of transcriptional regulation indicate that the rate of GM-CSF mRNA transcription is increased, while the elevated TGFα is likely the result of message stabilization. While a number of cytokine regulatory networks exist in the skin, studies utilizing neutralizing antibodies against the growth factors of interest indicate that inhibition of the arsenic-induced increase in TGFα results in a corresponding decrease in the gene expression and secretion of GM-CSF. The present studies demonstrate that growth-promoting cytokines and growth factors are induced in keratinocytes following treatment with arsenic and could play a significant role in arsenic-induced skin cancer. © 1996 Academic Press, Inc.

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 (Sommers and McManus, 1953; Nriagu, 1994). Less severe dermatological effects, including hyperpigmentation and hyperkeratosis, appear on the palms and soles following arsenic exposure (Chen *et al.*, 1985). Epidemiological studies in humans have suggested that the population cancer risk due to arsenic in U.S. water supplies may be comparable to that of environmental tobacco smoke and radon in homes with risk estimates of around 1 per 1000 (Smith *et al.*, 1992). In contrast to other chemical carcinogens in humans, the mechanisms involved in tumor formation by arsenic are not well established. Exposure to arsenic has been shown to induce genes involved in a variety of cellular processes including proliferation, genetic recombination, and protective responses, such as the induction of heat shock proteins (Lee *et al.*, 1988; Edwards *et al.*, 1991; Stohrer, 1991).

In response to environmental stimuli, keratinocytes produce and secrete a number of inflammatory and chemotactic cytokines, such as interleukin-1α (IL-1α),[2] tumor necrosis factor-α (TNFα), and IL-8, as well as cellular growth factors including transforming growth factor-α (TGFα) and granulocyte macrophage-colony stimulating factor (GM-CSF). Overexpression of these mediators has been implicated in various pathological processes such as contact hypersensitivity, psoriasis, and neoplasia (Gottlieb *et al.*, 1988; Luger and Schwarz, 1990; Vasunia *et al.*, 1994). In particular, overexpression of TGFα has been associated with 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 (Gottlieb *et al.*, 1988; Imamoto *et al.*, 1991). When injected into initiated mouse

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[2] Abbreviations used: GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; NHEK, normal human epidermal keratinocytes; PCR, polymerase chain reaction; TGF, transforming growth factor; TNF, tumor necrosis factor.

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skin, TGFα induces DNA synthesis in epidermal cells (Furstenberger *et al.*, 1989) and keratinocytes transfected with a constitutive TGFα transgene develop benign skin papillomas when grafted to nude mice (Finzi *et al.*, 1988). Using transgenic mice, it was shown recently that targeted overexpression of TGFα to the epidermis elicits hyperplasia, hyperkeratosis, and spontaneous squamous cell carcinomas (Vassar and Fuchs, 1991; Dominey *et al.*, 1993). Furthermore, TGFα transgenic mice exhibit keratinocyte hyperproliferation and neoplasias in the pancreas, liver, and mammary epithelia (Jhappan *et al.*, 1990; Matsui *et al.*, 1990; Sandgren *et al.*, 1990), as well as an accelerated response to chemical-induced cancers (Takagi *et al.*, 1993). This suggests that TGFα overexpression has the unique ability to complement both initiation and promotion by serving as a tumor enhancer. In these studies we examine the hypothesis that arsenic induces inflammation and/or cell proliferation, which may ultimately lead to tumorigenesis, by modulation of keratinocyte-derived inflammatory or growth promoting cytokines, respectively.

MATERIALS AND METHODS

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) 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 Corp.). NHEK in passages 1–3 were subcultured at seeding densities of 2500–20,000 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 TGFα). The cells were allowed to grow for an additional 24 hr and fresh KGM lacking hydrocortisone and bovine pituitary extract containing sodium arsenite (Sigma Chemical Co., St. Louis, MO), phorbol myristate acetate (PMA; Sigma), or selected recombinant cytokines was added. Recombinant human GM-CSF, TGFα, and IL-1α were obtained from R & D Systems (Minneapolis, MN). For antibody studies the keratinocytes were incubated for 30 min with neutralizing antibodies to either GM-CSF or TGFα (R & D Systems) prior to the addition of sodium arsenite. The cultures were allowed to incubate for an additional 2 to 18 hr and the cells were collected for RNA isolation or the supernatants were 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 (Wilmer *et al.*, 1994). Keratinocytes from two different donors were examined in these studies, and while the profiles of cytokine secretion and gene expression were qualitatively identical after arsenic treatment, quantitative differences, as determined by culture viability, were apparent. To compensate for this difference in sensitivity, arsenic exposure concentrations were adjusted so that the highest dose used for each donor had little or no effect on cell viability. For proliferation studies, cells were seeded in KGM in 25-cm^2 tissue culture flasks and allowed to establish for 24 hr. Triplicate cultures were treated with varying concentrations of sodium arsenite and allowed to grow to approximately 75% confluency. 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. For thymidine incorporation studies, 1.5 × 10^3 cells/well were cultured in 96-well U-bottom culture plates (Costar, Cambridge, MA) for 48 hr in KGM and then treated with varying concentrations of sodium arsenite. After 24 hr 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 hr. 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). [^{3}H]Thymidine incorporation was quantitated by liquid scintillation counting.

Cytokine secretion. GM-CSF and IL-1α concentrations were determined by ELISA using commercially available systems (Genzyme, Cambridge, MA) and TNFα concentrations measured by a cytolytic assay using L929 fibroblast cells treated with 5 μg/ml actinomycin D (Sigma; Beutler *et al.*, 1985). Results are expressed as the mean for quadruplicate determinations from one of three representative experiments. Immunoreactive TGFα 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. TGFα was visualized by using a 1:100 dilution of polyclonal goat anti-human TGFα antibody (R & D Systems) 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, Piscataway, NJ).

mRNA extraction and RT–PCR. Cultured normal human keratinocytes were collected, homogenized in a small volume of RNAzol B solution (Biotecx Laboratories, Inc., Houston, TX), and total cellular RNA was extracted according to the manufacturer's 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(dT) 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(dT) 12-18 primer (BRL, Gaithersburg, MD). After the reaction mixture reached 42°C, 400 U SuperScript 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 TGFα, TNFα, IL-1α, GM-CSF, *c-myc*, β-actin, and G3PDH mRNAs were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The primer sequences and sizes of amplified products are as follows: Human TGFα, 5′ ATGGTCCCC-TCGGCTGGACAG 3′, 5′ GGCCTGCTTCTTGTGGCTGGCA 3′, amplified PCR fragment, 297 bp; human IL-1α, 5′ CAAGGAGAGCATGGT-GGTAGTAGCAACCAACG 3′, 5′ TAGTGCCGTGAGTTTCCCAGA-AGAAGAGGAGG 3′, amplified PCR fragment, 491 bp; human TNFα, 5′ GAGTGACAAGCCTGTAGCCCATGTTGTAGCA 3′, 5′ GCAATGATC-CCAAAGTAGACCTGCCCAGAC 3′, amplified PCR fragment, 444 bp; human GM-CSF, 5′ ATGTGGCTGCAGAGCCTGCTGC 3′, 5′ CTGGCT-CCCAGCAGTCAAAGGG 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′ TGAAGGTCGGAGTCAACGGAT-TTGGT 3′, 5′ CATGTGGGCCATGAGGTCCACCAC 3′, amplified PCR fragment, 983 bp. Five-microliter aliquots of the synthesized cDNA (corresponding to 10 ng of mRNA) were added to 45 μl of PCR mix containing 5 μl of 10X 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 an 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°C for 30 sec, 55°C for 30 sec, and 55°C for 1 min using a 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 semiquantitative analysis of signal strength as previously described (Wilmer *et al.*, 1994). For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 33, and 35 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength (Kayama *et al.*, 1995). 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 using the Eagle Eye II Image analysis system (Stratagene, La Jolla, CA). Area under the curve, normalized for G3PDH content, was determined by using NIH Image 1.54 analysis software.

Competitive PCR. Quantitative analysis of GM-CSF mRNA transcripts was performed by competitive PCR using serial dilutions of cDNA fragments containing complementary sequences for the GM-CSF primer (PCR MIMICs; Clontech). Each primer set is used to amplify both a target gene cDNA and a MIMIC fragment which is of a different size than the target cDNA. Aliquots of sample cDNA were amplified for 35 cycles in the presence of respective competitors ranging in concentration from 3×10^{-2} to 3×10^{-5} attomoles of competitor. The ratio of the intensities of the target band to the competing band was plotted against known competitor concentrations present in each reaction. The concentration where the ratio is approximately one represents the amount of target mRNA transcripts in the initial sample and was derived by linear regression analysis.

Message stabilization. Keratinocytes were cultured as described above to approximately 65% confluency. After a 24-hr incubation in KGM without hydrocortisone the medium was removed and fresh medium containing 5 μg/ml actinomycin D ± 0.5 μM sodium arsenite was added. At selected time points the cells were collected, the mRNA was isolated as described above, and 15 μg/sample was loaded onto a denaturing gel and separated by electrophoresis. The mRNA was transferred to nylon filters (GeneScreen Plus; Dupont NEN, Boston, MA) by capillary blotting in 20X SSC (1X SSC, 0.15 M NaCl, 15 mM sodium citrate) and when transfer was complete, the filters were baked in a vacuum oven at 80°C for 1 hr. Filters were prehybridized at 42°C overnight in a solution of 50% formamide, 5X Denhardt's solution, 5X SSC, 1.0% SDS, 10X dextran sulfate, and 100 μg/ml salmon sperm DNA. Plasmids containing DNA for TGFα were obtained from ATCC (Rockville, MD) and the linearized DNA was labeled with [^{32}P]dCTP via nick translation using a commercially available kit (Boehringer Mannheim, Indianapolis, IN). The human GM-CSF cDNA probe was purchased from Clontech and labeled as described above. Filters were probed for TGFα or GM-CSF mRNA overnight and washed four times in 2X SSC, 0.1% SDS (1× RT for 15 min, 3× 55°C for 30 min). This was followed by a stringent wash in 0.1X SSC, 0.1% SDS for 30 min at 55°C. The filters were autoradiographed (XAR5 film; Eastman Kodak, Rochester, NY) and the films were analyzed as described above. Filters were washed for 35 min in 0.1X SSC, 0.1% SDS for 45 min at 90°C and reprobed with a ^{32}P-labeled G3PDH cDNA probe (Clontech).

Nuclear runoff assay. Keratinocytes were cultured as described above to approximately 65% confluency. After a 24-hr incubation in KGM without hydrocortisone the medium was removed and fresh medium containing either 0.2 μM sodium arsenite or 10 nM PMA was added. The cells were incubated 4 hr and the nuclear runoff transcription assay was performed as previously described (Gaido *et al.*, 1992). The nascent transcripts were processed for hybridization by the hot phenol method of Nevins (1987). An equal amount of radiolabeled RNA was added to DNA immobilized on nylon filters and hybridized for 3 days at 42°C in 50% formamide, 5X SSPE (1X SSPE, 0.15 M sodium chloride, 0.01 M sodium phosphate, 1 mM EDTA), 5X Denhardt's, 0.5% SDS, and 100 μg/ml salmon sperm DNA. Filters were prepared by adding 5 μg alkali-denatured plasmid per slot and UV cross-linking (UV Stratalinker; Stratagene, La Jolla, CA). After incubation the filters were washed, autoradiographed, and quantitated by image analysis as described above.

Statistical analysis. Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 Multicomparison (Bolt, Beranek and Newman, Cambridge, MA) procedure using the 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 $p < 0.05$.

RESULTS

Initial studies examined the effect of arsenic exposure on cytokine gene expression in normal human epidermal keratinocytes. Poly(A)$^+$ mRNA isolated from arsenic-treated keratinocytes was examined by RT–PCR and the constitutively expressed genes, G3PDH and β-actin, were used to adjust for differences in mRNA concentrations. As shown in Fig. 1, within 4 hr following the addition of arsenic to keratinocyte cultures, increases in GM-CSF, TNFα, and TGFα mRNA transcripts were evident. In the case of TNFα, the increases in mRNA transcripts appeared to be dose-dependent. In some experiments PMA (10 nM) was included as a positive control. Consistent with the increase in TGFα and GM-CSF transcripts, suggesting an elevated growth factor response, *c-myc* protooncogene expression was also elevated following arsenic treatment (Fig. 1). The expression of *c-myc* is associated with keratinocyte cell proliferation (Pietenpol *et al.*, 1990) and increased expression suggests that growth factor secretion induced by arsenic accompanied cell proliferation. To confirm this, cell proliferation, as measured by quantitating cell numbers and as suggested by incorporation of [^{3}H]thymidine, was examined in keratinocyte cultures treated with sodium arsenite. Low levels of sodium arsenite (0.001–0.005 μM) produced a modest but significant increase in keratinocyte proliferation (Figs. 2A and 2B), while higher levels of arsenic (0.1–4 μM), associated with cytotoxicity, prevented cell growth and inhibited [^{3}H]-thymidine incorporation (data not shown).

To provide quantitative values for changes in mRNA expression of specific growth factors, GM-CSF mRNA levels were determined in a competitive PCR assay (Fig. 3). Consistent with our initial studies, there was no constitutive expression of GM-CSF in control cell cultures. GM-CSF mRNA levels were elevated after treatment with 4 μM arsenic. Regression analysis using the GM-CSF competitors showed equal intensity of competitive and target bands in lane 6, corresponding to an mRNA concentration of approximately 3.3×10^{-3} attomoles.

Cell viability, as assessed by trypan blue dye exclusion,

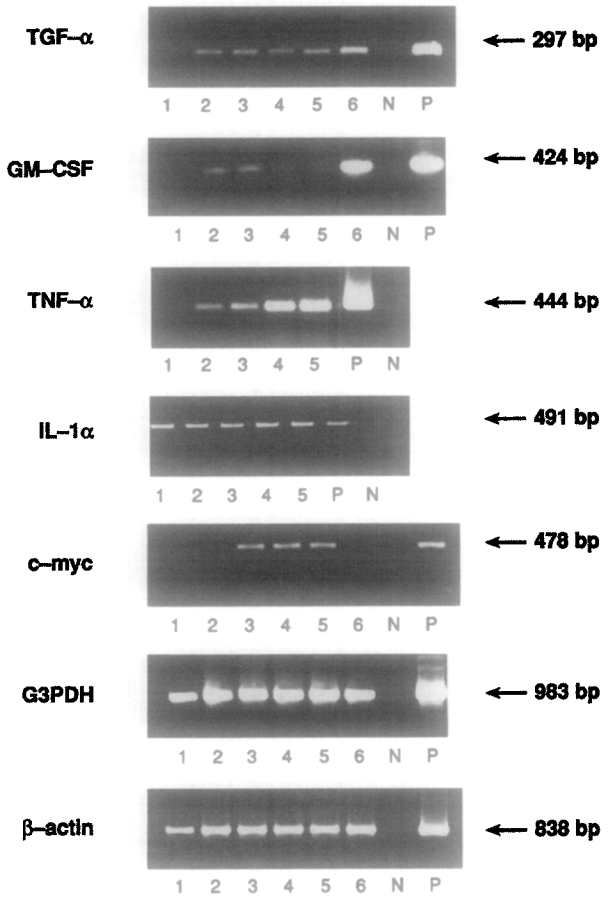

FIG. 1. Amplification of TGFα, GM-CSF, *c-myc*, IL-1α, TNFα, G3PDH, and β-actin mRNAs by RT–PCR. Normal human epidermal keratinocytes were exposed to sodium arsenite or 10 nM PMA for 4 hr and total RNA was isolated as described under Materials and Methods. RNA concentrations and PCR cycles were titrated to establish standard curves, to document linearity, and to permit semiquantitative 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.

indicated that only arsenite concentrations above 8 μM affected cell viability in this keratinocyte donor following 18 hr of culture (<12% decrease at 8 μM; Fig. 4A). It was then determined whether the increased levels of cytokine mRNA expression reflected translation and cytokine secretion in keratinocyte cultures. At noncytotoxic concentrations, sodium arsenite induced a dose-dependent increase in secreted GM-CSF and TNFα (Fig. 4B) as well as immunoreactive TGFα (Figs. 4C and 4D) within 18 hr following addition to the keratinocyte cultures. Neither proinflammatory cytokines, such as IL-6 (data not shown) or IL-1α (Fig. 4B), nor chemokines, including IL-8 or MCP-1 (data not shown), were secreted in response to arsenic.

Increases in mRNA transcripts may occur via increases in transcriptional activity or accumulation of transcripts due to defects in the regulation of enzymes which promote mRNA degradation. To examine whether the increased concentrations of growth factors in culture supernatants were due to increased synthesis, we examined the effects of sodium arsenite on cytokine mRNA transcription rates in a nuclear runoff assay. Image analysis of nuclear runoff studies conducted 4 hr following arsenic treatment indicated that transcription of GM-CSF was induced approximately threefold by arsenic, while transcription of TGFα was unchanged when compared to untreated cells (Fig. 5). Interestingly, the rate of TGFβ2 mRNA transcription was decreased 35% in arsenic-treated cultures. As the increases in TGFα mRNA

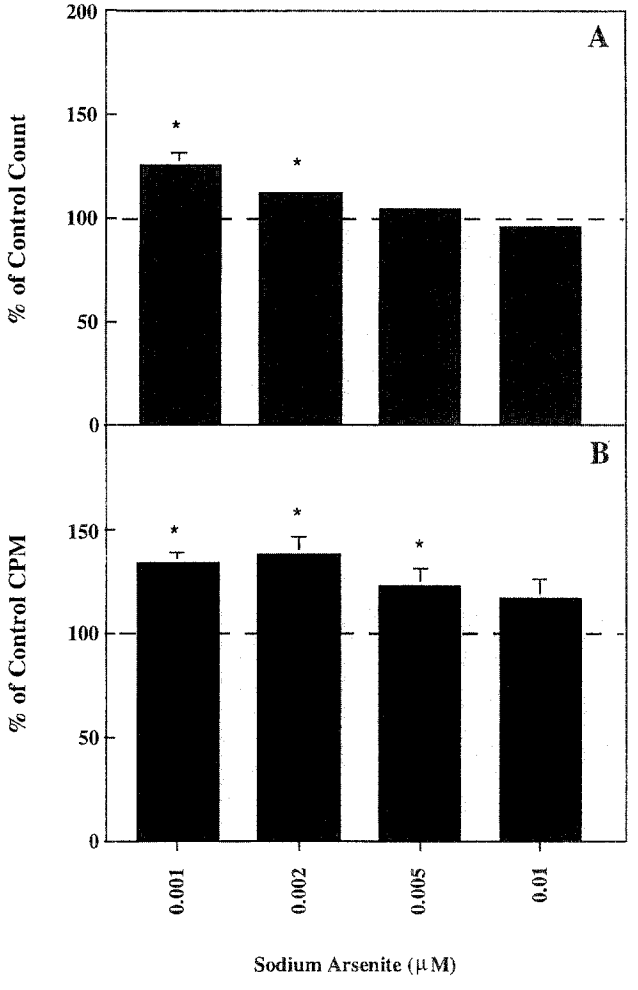

FIG. 2. Stimulation of cell proliferation in keratinocytes by sodium arsenite. Keratinocytes were cultured in 24-cm^2 culture flasks or 96-well U-bottom culture plates as described under Materials and Methods and then treated with varying concentrations of sodium arsenite. (A) Triplicate cultures of 25-cm^2 flasks were used for determination of actual cell numbers. Cell viability was determined by trypan blue exclusion. Each bar represents the mean percentage of viable control cell counts ± SE. (B) Twelve wells were used for each treatment for thymidine incorporation. The cells were harvested and incorporated radiolabel quantitated as described under Materials and Methods. Each bar represents the mean percentage of control CPM ± SE from three replicate experiments. *Significantly different from control cultures at $p < 0.05$.

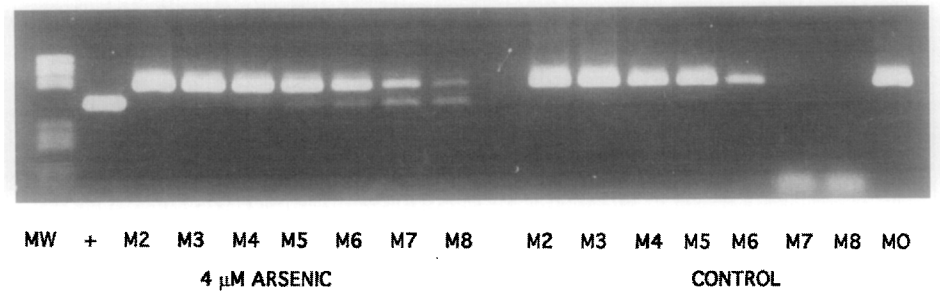

FIG. 3. Quantitative analysis of arsenic-induced changes in GM-CSF mRNA levels by competitive PCR. Normal human epidermal keratinocytes were exposed to 4 μM sodium arsenite for 4 hr and total RNA was isolated as described under Materials and Methods. The competitive PCR was performed as described under Materials and Methods and subjected to 35 cycles of PCR amplification. M2–M8 denote serial 3-fold dilutions of the MIMIC competitor (M2 = 3.0×10^{-1} attomoles, M3 = 1.0×10^{-1} attomoles, M4 = 3.3×10^{-2} attomoles, M5 = 1.1×10^{-2} attomoles, M6 = 3.3×10^{-3} attomoles, M7 = 1.1×10^{-3} attomoles, M8 = 3.3×10^{-4} attomoles). The top band contains the GM-CSF PCR product (424 bp) while the bottom band contains the MIMIC competitor (440 bp).

levels were not due to changes in the rate of transcription, the effect of sodium arsenite treatment on TGFα mRNA stabilization was examined. As shown in Fig. 6, treatment with 0.5 μM sodium arsenite, in the presence of actinomycin D, resulted in an initial elevation of TGFα mRNA, which returned to baseline by 6 hr, while levels in control cultures decreased 40% within 1 hr and were only 10% of baseline levels at 6 hr.

It is well established that similar to proinflammatory cytokines, growth promoting cytokines have both autocrine and paracrine activities. To help determine the regulatory relationship between the induction of these cytokines in arsenic exposure, secretion of growth promoting cytokines was examined following the addition of anti-cytokine antibodies to keratinocyte cultures. Addition of anti-TGFα antibodies to keratinocyte cultures inhibited arsenic-induced increases in GM-CSF mRNA expression (data not shown) and secretion of GM-CSF protein (Fig. 7). Addition of anti-TGFα antibodies did not alter the transcription or secretion of TNFα, suggesting a GM-CSF-specific effect. Antibodies to GM-CSF had little, if any, effect on arsenic-induced increases in TGFα message (data not shown). In many cells the effects of positive growth regulators such as TGFα are counterbalanced by the effects of TGFβs. Although transcription of TGFβ2 mRNA was downregulated by arsenic treatment (see Fig. 5), addition of TGFβ2, alone, to keratinocyte cultures increased the expression of both GM-CSF and TGFα mRNA compared to control cultures, and this increase was unchanged in the presence of sodium arsenite (Fig. 8). Thus, while TGFβ2 may have regulatory effects in keratinocytes, as evidenced by its ability to increase mRNA levels for particular cytokines, it appears that the arsenic-induced decrease in TGFβ2 is independent of its induction of GM-CSF and TGFα, while induction of GM-CSF by arsenic appears to be dependent upon TGFα.

DISCUSSION

The present studies have demonstrated differential alterations in gene expression and secretion of growth factors, including GM-CSF, TGFβ, TNFα, and TGFα in primary human epidermal keratinocytes cultured in the presence of low micromolar concentrations of sodium arsenite. These changes were accompanied by a moderate increase in cell proliferation as indicated by increases in *c-myc* gene expression, number of cell recovered at 75% confluency, and incorporation of [^{3}H]thymidine into cellular DNA. Studies of transcriptional regulation indicate that while the rate of GM-CSF mRNA transcription is increased, the elevation in TGFα mRNA levels is likely the result of message stabilization. To explain how arsenic might affect cytokine regulatory networks, we examined the autocrine and paracrine relationships between GM-CSF, TGFα, and TGFβ2 in keratinocytes after treatment with sodium arsenite. Of particular importance, arsenic-induced GM-CSF secretion was significantly reduced after pretreatment with antibodies to TGFα, suggesting that TGFα helps regulate GM-CSF expression. In contrast, antibodies to GM-CSF had little effect on the arsenic-induced increases in TGFα mRNA expression. Although sodium arsenite caused a significant decrease in TGFβ2 mRNA expression, addition of TGFβ2 to keratinocytes in the presence of arsenic did not affect the expression of either arsenic-induced GM-CSF or arsenic-induced TGFα mRNA, indicating that TGFβ2 is not involved in this network. Thus arsenic may stimulate keratinocyte proliferation by multiple mechanisms including direct effects on gene expression, as well as indirectly through the modulation of cytokine regulatory networks.

The cytokine pattern produced by keratinocytes in response to arsenic was unlike previous studies with contact irritants and contact sensitizers where secretion of proinflammatory cytokines, such as IL-1β, and chemokines, such

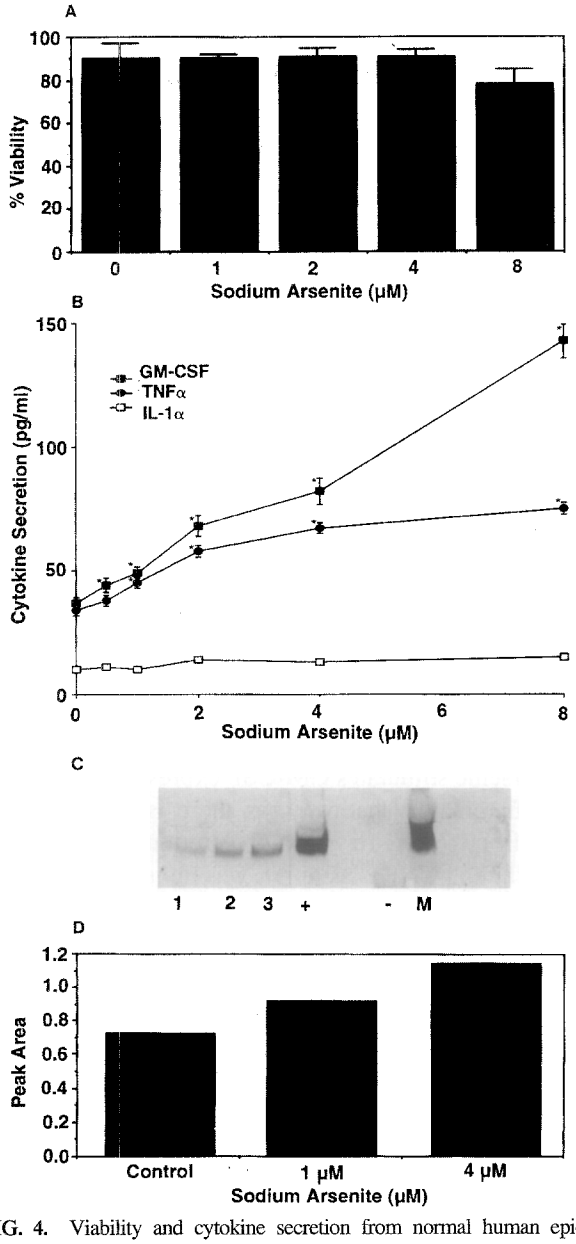

FIG. 4. Viability and cytokine secretion from normal human epidermal keratinocytes grown in the presence of arsenite for 18 hr. (A) Cell viability as determined by Trypan blue exclusion. (B) GM-CSF and IL-1α concentrations were determined by ELISA using commercially available systems (Genzyme, Cambridge, MA) and TNFα 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. *Significantly different from medium control at $p < 0.05$. (C) Immunoreactive TGFα as determined from culture supernatants by immunoblotting. TGFα 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; +, TGFα std; −, water control; M, 30-kDa molecular weight standard. (D) Computerized laser densitometer quantitation of peak area of lanes 1–3 of C.

as IL-8, rather than growth factors predominated (Wilmer *et al.*, 1994). TGFα and to a slight extent GM-CSF are expressed constitutively in normal skin although the latter is not found in unstimulated cultured keratinocytes (Luger and Schwarz, 1990; Vasuina *et al.*, 1994; Wilmer *et al.*, 1994). GM-CSF can participate in inflammatory reactions and can be induced in keratinocytes by a number of cytokines, including IL-2, EGF/TGFα, and TNFα (Ansel *et al.*, 1990; Owsianowski *et al.*, 1991). GM-CSF is also a growth factor for keratinocytes (Hancock *et al.*, 1988; Braunstein *et al.*, 1994) and has been implicated in tumor promotion via mediating inflammatory cell influx and increasing dark cell numbers in mouse skin (Vasunia *et al.*, 1994). GM-CSF is actively transcribed during the tumor promotion process, being involved in leukocyte migration and activition (Robertson *et al.*, 1994).

TGFα, like GM-CSF, is a growth factor for keratinocytes (Coffey *et al.*, 1987; Olaniran *et al.*, 1995) and increased levels of TGFα have been associated with a number of diseases characterized by hyperkeratosis, such as psoriasis (Elder *et al.*, 1989). In humans, chronic arsenic exposure results in hyperkeratosis, which appears most frequently on the palms of the hands and soles of the feet (Chen *et al.*, 1985). Addition of exogenous TGFα to keratinocyte cultures results in patterns of cytokeratin expression, including upregulation of K8 and K18 and suppression of K1, similar to those observed after oncogenic transformation (Cheng *et al.*, 1993). Keratinocytes cultured with tumor promoting agents, including phorbol esters or epigenetic carcinogens such as TCDD, or transfected with the v-Ha-*ras* oncogene also overexpress TGFα mRNAs and secrete increased TGFα, suggesting a role for TGFα in the growth and development of skin neoplasias (Choi *et al.*, 1991; Glick *et al.*, 1991; Pittelkow *et al.*, 1991; Gaido *et al.*, 1992). This is supported by studies showing that nude mice develop benign skin papillomas when engrafted with keratinocytes transfected with the TGFα transgene (Finzi *et al.*, 1988). However, recent studies indicate that other EGF receptor ligands, such as amphiregulin, heparin-binding EGF-like growth factor, and betacellulin, are also upregulated in v-Ha-*ras*-induced neoplasia and that TGFα may not be essential (Dlugosz *et al.*, 1995). When injected into initiated mouse skin, TGFα induces DNA synthesis in epidermal cells, an activity analogous to early stages in tumor formation (Furstenberger *et al.*, 1989). As the present studies demonstrate that arsenic induces overexpression of TGFα and GM-CSF, resulting in keratinocyte proliferation, it can be hypothesized that *in vivo* exposure to arsenic leads to enhanced proliferation of initiated cells and increases in tumor formation.

A number of dermal cytokine regulatory networks are active in both physiological and pathological processes in the skin. One pathway, involved in inflammatory responses and wound healing, is regulated by expression of keratinocyte-derived IL-1α and TNFα. These primary cytokines act in an autocrine and paracrine fashion to stimulate increased production of other cytokines including GM-CSF and IL-6

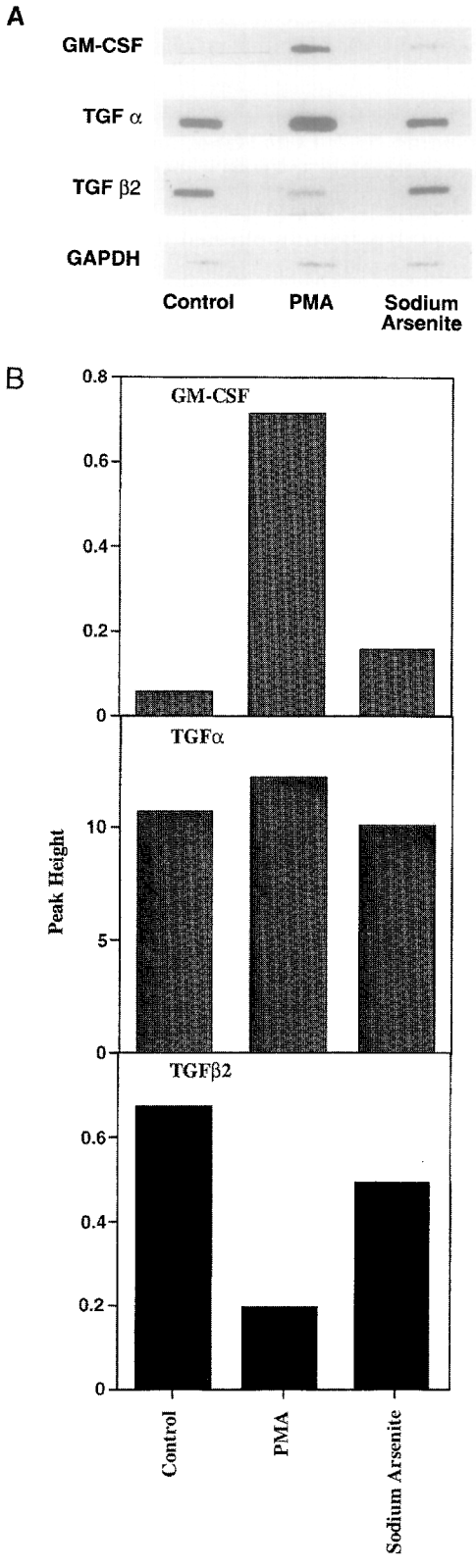

FIG. 5. Effect of sodium arsenite on GM-CSF, TGFα, and TGFβ2 transcription rates. Normal human epidermal keratinocytes were exposed to 0.5 μM sodium arsenite or 10 nM PMA for 4 hr, nuclei were collected, and the nuclear runoff analysis was performed as described under Materials and Methods. GAPDH was used as a control to load equal concentrations of RNA. (A) Slot blot of TGFα, GM-CSF, and TGFβ2 and GAPDH transcripts. (B) Image analysis of slot blots corrected for GAPDH levels.

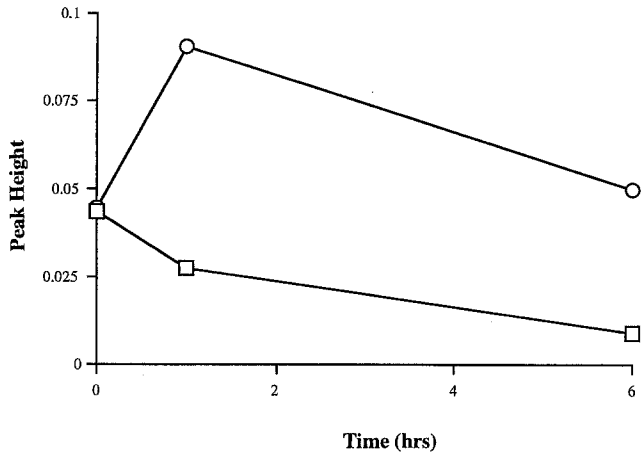

FIG. 6. Stabilization of TGFα mRNA by arsenic. Keratinocytes were cultured to 75% confluency and then incubated in medium containing 5 μg/ml actinomycin D (Sigma) ± 0.5 μM sodium arsenite. At the indicated time points cells were collected by scraping, and the mRNA was isolated for Northern analysis as described under Materials and Methods. Graphic representation of image analysis of a single experiment corrected for the G3PDH loading control. (□) Medium only; (○) 0.5 mM sodium arsenite.

(Ansel *et al.*, 1990; Partridge *et al.*, 1991). Our results indicate that arsenic stimulates GM-CSF expression independent of this inflammatory pathway, as there is no evidence of increases in either IL-1α or IL-6 secretion or mRNA transcripts, although TNFα secretion was enhanced. TNFα has been shown to stimulate cell proliferation in a number of cell types, including keratinocytes, hepatocytes, and thymocytes (Ranges *et al.*, 1988; Malejczyk *et al.*, 1992; Satoh and Yamazaki, 1993), and it has been suggested that its proliferative effects are a result of the ability of TNFα to induce the paracrine synthesis and secretion of additional cytokines (Matsue *et al.*, 1993; Kubo *et al.*, 1996). Changes in expression of TGFα in keratinocytes may occur via activation of an autocrine loop, which may be influenced by a number of cytokines including IL-1α, IL-1β, TNFα, and IFNγ (Elder *et al.*, 1989; Lee *et al.*, 1991; Nickoloff *et al.*, 1991). The studies described herein suggest that TGFα may play a role in regulating GM-CSF transcription, as neutralizing antibodies to this cytokine abrogated arsenic-induced GM-CSF secretion.

There are a number of members of the TGFβ family. These have multiple functions in the skin, which are dependent on the target cell type, culture conditions, and the presence of other biologically active peptides. TGFβs inhibit proliferation in cultured mouse and human keratinocytes and may act via inhibition of *c-myc* transcription (Shipley *et al.*,

1986; Pietenpol *et al.*, 1990). In numerous cell types the antiproliferative effects of TGF-β may be counteracted by the overexpression of positive growth regulators such as TGFα (Edwards and Heath, 1991). Our studies indicate that arsenic serves to influence both positive (TGFα, GM-CSF) and negative (TGFβ2) regulators of keratinocyte growth by stimulating the former and inhibiting the latter. However, it appears that these pathways are independently controlled, as adding TGFβ to arsenic-treated cultures does not alter the expression of either TGFα or GM-CSF.

The high affinity of arsenic for sulfhydryl groups leads to its accumulation and retention in keratin-rich cells such as epidermal keratinocytes, likely making these cells a sensitive target for arsenic-induced toxicity (Molin and Wester, 1976). Arsenic has been shown to alter the differentiation process in keratinocytes by multiple mechanisms, including suppression of involucrin expression, increases in tyrosine phosphorylation, and alterations in AP-2 transcription factor activity (Kachinskas *et al.*, 1994). In addition, arsenic has been shown to affect cellular processes in other cell types by varying mechanisms, including direct alterations in expression of certain genes, such as those which regulate the expression of heat shock proteins and transcriptional activators such as *c-fos* (Van Wijk *et al.*, 1993; Wijeweera *et al.*, 1995), as well as indirectly via changes in DNA methylation resultant from modification of levels of methyl donor pools and inhibition of methyltransferase activity (Mass and Wang,

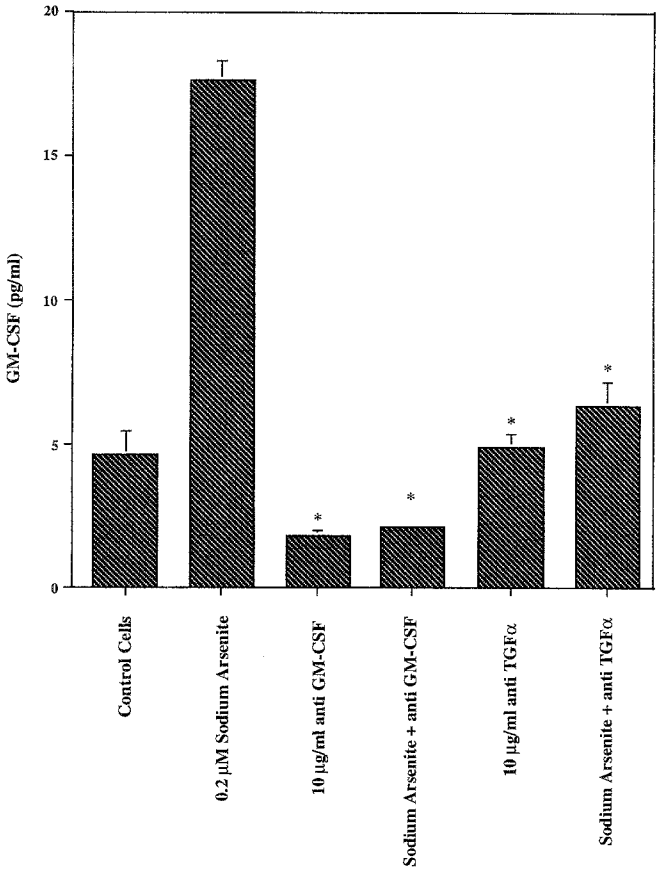

FIG. 7. Effects of anti-GM-CSF and anti-TGFα antibodies on the arsenic-induced increase in GM-CSF secretion. Normal human epidermal keratinocytes were cultured to 75% confluency as described under Materials and Methods and then exposed to 0.2 μM sodium arsenite $\pm$ 10 μg/ml anti-GM-CSF or 10 μg/ml anti-TGFα for 24 hr. The supernatants were harvested and GM-CSF secretion was quantitated by ELISA assay. Each bar represents the mean $\pm$ SE of triplicate cultures. *Significantly different from medium control at $p < 0.05$.

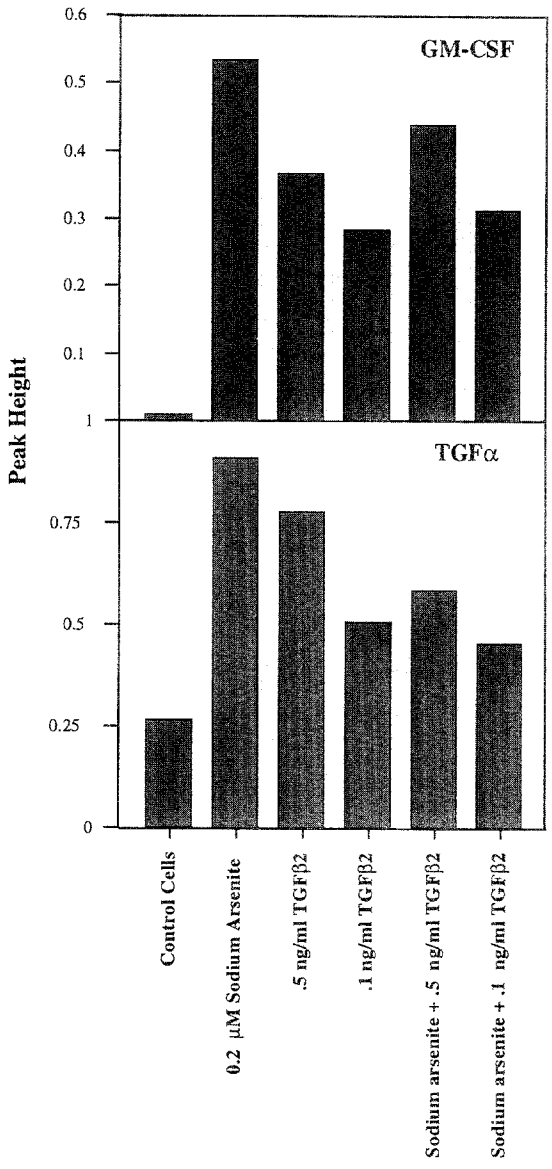

FIG. 8. Effects of TGFβ2 on arsenic-induced GM-CSF and TGFα mRNA expression. Normal human epidermal keratinocytes were exposed to 0.2 μM sodium arsenite and 0.1 or 0.5 ng/ml TGFβ2 or combinations thereof for 4 hr and total RNA was isolated and RT–PCR performed as described under Materials and Methods. Graphic representation of image analysis of a single experiment corrected for the G3PDH loading control.

1996). In the latter studies, the investigators have shown that arsenic increases the level of 5-methylcytosine residues in the promoter region of the *p53* gene in a human lung epithelial cell line. While the effects of arsenic on DNA methylation of the *p53* gene have not been examined specifically in keratinocytes, studies comparing methylation of CpG sites in the *p53* gene in a wide variety of human tissues, including keratinocytes, fibroblasts, and epidermal cells, have indicated that methylation of this gene is tissue-independent (Tornaletti and Pfeifer, 1995), suggesting that arsenic may induce alterations in DNA methylation in a number of tissues.

In conclusion, arsenic, a ubiquitous element, represents a human health concern when concentrated in the environment from natural or anthropogenic processes. Arsenic toxicity is highly dependent on its chemical form, with inorganic arsenite (iAs^{III}) and arsenate (iAs^{V}) more acutely toxic than the methylated organic species (Yamauchi and Fowler, 1994). It is well established that chronic exposure to inorganic arsenic in drinking water is associated with increased mortality from skin and lung cancers and recent studies have linked arsenic exposure to neoplasias in a number of internal organs, including liver, bladder, kidney, and prostate (EPA, 1987; Smith *et al.,* 1992; Nriagu, 1994; Chiou *et al.,* 1995; Tsuda *et al.,* 1995). 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, arsenic-exposed pesticide workers, and residents of areas where arsenic-contaminated drinking water has been found (Sommers and McManus, 1953; EPA, 1987; Chen and Lin, 1994; Nriagu, 1994). 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 (reviewed in EPA, 1987). Considering the accumulating evidence that keratinocyte growth factors enhance skin tumor formation, the present data suggest that arsenic may exert its influence on carcinogenicity via the stimulation of keratinocyte-derived growth factors rather than genetic events.

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