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Mechanisms of Arsenic Carcinogenicity: Genetic or Epigenetic Mechanisms?

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Environmental and occupational exposure to arsenic is associated with increased risk of skin, urinary bladder, and respiratory tract cancers. The mechanisms responsible for arsenic carcinogenesis have not been established. Arsenic does not act through classic genotoxic and mutagenic mechanisms, as do other metals such as cadmium or chromium. Increasing evidence indicates that arsenic acts at the level of tumor promotion by modulating the signaling pathways responsible for cell growth.

KEY WORDS: gene expression, reactive oxygen species, skin cancer, bladder cancer.

Epidemiological Evidence

Exposure to trivalent and pentavalent forms of arsenic occur worldwide through environmental and occupational exposures. Epidemiologic studies have demonstrated that exposure to inorganic arsenic is associated with increased risk of human cancer of the skin, urinary bladder, respiratory tract, liver, and kidney.[1-4] For example, arsenic contamination of water supplies has resulted in very high incidences of skin lesions and skin cancer in exposed populations from Taiwan, China, Bangladesh, the Southwestern United States, and Central and South America. Arsenic-induced skin cancers occur in sun-exposed as well as -unexposed areas and include either squamous cell carcinomas, basal cell carcinomas, or combined lesions.[5,6] Additional manifestations of chronic arsenic dermatotoxicity include hyperpigmentation and hyperkeratosis.[7] The association between arsenic exposure and urinary bladder. cancers, typically transitional cell carcinomas, has been observed in the same endemic areas of the world where skin cancer populations were identified. Inhalation of arsenic, mainly from occupational exposures, has also been related to increased cancer risk, primarily lung cancer.[8] On the basis of numerous epidemiological studies, arsenic has been classified as a strong human carcinogen, and population cancer risk due to arsenic has been suggested to be comparable to environmental tobacco smoke and radon in homes with risk estimates of around 1 per 1000.[9] The EPA estimates that over 350,000 people in the U.S. consume drinking water containing over 50 μg/L of arsenic—the current EPA standard—and more than 2.5 million people use water containing more than 25 μg/L of arsenic.[3,10] Subsequently, there has been significant regulatory pressure to lower the acceptable levels.

Genetic Mechanisms of Carcinogenicity

Although several hypotheses have been proposed, the mechanisms responsible for arsenic carcinogenesis have not been established, partly because carcinogenesis in rodent models has never been convincingly demonstrated. Arsenic fails to induce mutations in bacterial or Chinese hamster cells[11,12] and causes chromatid abnormalities, such as sister chromatid exchanges, only at high

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concentrations.[13,14] Arsenic has been demonstrated to induce amplification of dihydrofolate reductase gene in mouse 3T6 cells, and gene amplification has been suggested as a possible mechanism of arsenic carcinogenicity.[15] DNA repair enzymes are inhibited by arsenic resulting in a comutagenic response with X-rays, ultraviolet radiation, or alkylating agents,[16] although this mechanism has not been confirmed by epidemiological data. Because the concentrations of arsenite required to inhibit DNA ligase activity in vitro are higher than that needed to inhibit repair within cells, it has been argued that arsenic may modulate the control of cellular DNA repair processes. In vitro studies have focused on the ability of arsenic to affect DNA repair as a result of alterations in DNA methylation patterns.[17,18] Arsenic is enzymatically methylated primarily in the liver and requires cofactors, such as S-adenosyl-methionine (SAM) and methyltransferases, which may be involved in DNA methylation. Exposure of liver epithelial cells to arsenic in vitro induced malignant transformation in parallel with DNA hypomethylation.[17] A549 cells, a type II lung epithelial cell line, cultured in the presence of arsenic causes a dose-responsive hypermethylation within 341-base pair fragment of the promoter of the cell cycle regulator molecule p53.[18] The relevance of these findings to arsenic exposure in humans or experimental animals has yet to be evaluated.

Epigenetic Mechanisms of Carcinogenicity

Increasing evidence supports the hypothesis that arsenic shares many properties of tumor promoters by affecting specific cell signal transduction pathways involved in cell proliferation. Similar to classic tumor promoters, such as PMA, okadaic acid, and UV light, trivalent arsenic activates transcription factors, such as AP-1, and induces immediate early genes including *c-fos, c-jun* and *c-myc*,[19-21] whose products stimulate cell proliferation. Consistent with these observations, arsenic induces a moderate but persistent increase in keratinocyte cell proliferation in vitro, as evidenced by increases in thymidine incorporation,[22] cell cycling,[23] labeling of Ki-67 (a proliferating cell marker),[23] and ornithine decarboxylase activity.[24]

Recently, we demonstrated that a human urinary bladder epithelial cell line also responds to arsenic by moderately enhanced cell growth.[25] Histological examination and PCNA immunostaining established that hyperplasia occurred in urinary bladder epithelial cells and epidermis of mice following in vivo exposure to arsenite.[22,25] The growth-stimulatory effect of arsenic in keratinocytes and uroepithelial cells is accompanied by increased AP-1 DNA binding and up-regulation of *c-fos* and *c-jun* gene expression.[20,25] Exposure of mice to arsenite in drinking water also induced AP-1-DNA binding in urinary bladder. This binding was characterized as functional since it resulted in an increase in AP-1-luciferase reporter activity in transgenic mice. Characterization of arsenic-induced AP-1 DNA binding complex demonstrated that the complex consisted of *c-Jun/c-Fos* heterodimers, which is a common heterodimer responsible for regulating cell mitogenesis.[26] Of particular relevance to these studies is that *c-Jun* expression has been reported as a concomitant factor of urinary bladder transitional carcinoma.[27,28]

The mechanisms by which arsenic activates transcription factors and genes may be explained by some of its physicochemical properties. Arsenic follows similar metabolic pathways in humans and laboratory animals, where pentavalent arsenic is first reduced to the trivalent form, which is subsequently methylated to monomethylarsenic acid (MMA) and then to dimethylarsenic acid (DMA).[29] The majority of evidence indicates that the inorganic forms, particularly iAs^{3+}, are responsible for the toxicity,[30] although there are several reports of toxic responses associated with methylated metabolites.[31,32] For example, exposure of rats to DMA for 97–104 weeks produced bladder tumors,[31] and uroepithelial toxicity was observed in rats administered 100 ppm of DMA in the feed.[32] The toxicity of arsenite has been related to its high reactivity with vicinal sulfhydryl groups on macromolecules such as glutathione (GSH) and cystein.[33] Arsenite accumulates in tissues rich in sulfhydryl-containing molecules, such as keratin,[34,35] which may explain the accumulation of arsenic in epithelial cells from the skin and bladder and development of carcinogenicity in these tissues.

At high cell concentrations, arsenite inhibits glutathione reductase activity and diminishes cellular levels of reduced GSH,[36] potentially resulting in an altered cellular redox state and oxidative stress. Oxidative stress is associated with gene expression through activation of oxidant-sensitive transcription factors, such as AP-1 and particularly, NF-κB.[37] In this respect, it has been shown that arsenic-induced *c-myc* expression, growth arrest, and DNA damage (GADD)153 gene

expression were increased after depletion of intracellular GSH levels by buthionine-sulfoximine (BSO) and suppressed in the presence of N-acetylcysteine (NAC), a precursor of GSH.[21,38] GSH, in addition to being an antioxidant, is involved in detoxification and methylation of arsenic by direct binding.[39] Arsenic-induced GADD153 expression, although inhibited by NAC, is not affected in the presence of reactive oxygen species (ROS) scavengers such as o-phenanthroline (a metal iron chelator) or mannitol (a hydroxyl radical scavenger), which inhibits H_2O_2-induced GADD153. This suggests that direct arsenic–GSH interactions, such as GSH reduction, are more likely involved in gene expression than induction by ROS. Recent studies reported a high frequency of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA damage, in arsenic-related skin cancers.[40] While this can be due to the generation of ROS, 8-OHdG can also be formed by direct electron transfer without participation of ROS. Further studies directly measuring generation of ROS by arsenic will be necessary to clarify the involvement of oxidative stress in arsenic toxicity.

Several studies have suggested that arsenic activates gene expression by modulation of intracellular phosphorylation events and mitogen-activated protein kinases (MAPK).[11,41-43] Cavigelli et al.[19] have demonstrated that arsenite, in contrast to arsenate, induces *c-Jun* N-terminal kinase (JNK) and p38 activation in HeLa cells, and that this occurs in parallel with AP-1 activation and *c-jun/c-fos* gene expression. It was suggested that arsenite interacts with the sulfhydryl groups on cysteine at the catalytic site of JNK phosphatase to inhibit its activity, resulting in prolonged JNK and p38 activation. A study conducted with PC12 cells, used commonly to study MAPK activation, has demonstrated that arsenite treatment potently activates both JNK and p38, and only moderately activates ERK. The activation of all 3 kinases by arsenic was prevented by the addition of NAC, suggesting a role of GSH and/or oxidative stress in the initiation of this response. In addition, it has been suggested that arsenite may induce ERK activation in PC12 cells by binding to the cysteine-rich domains of the epidermal growth factor receptor (EGFR) and subsequent activation of the Ras-dependent pathway.[41,42] Arsenic has been demonstrated to bind and modulate other receptors that have vicinal thiols in their binding sites, such as glucocorticoid receptors.[44] Recently, MAPK activation by arsenite has been shown to occur in JB6 mouse epidermal cell line, as evidenced by ERK phosphorylation and increased ERK activity at doses ranging from 0.8 to 200 μM.[43] Higher doses (> 50 μM) were required for JNK activation.[43] Furthermore, arsenite-induced cell transformation of this cell line was blocked by overexpression of a dominant negative ERK, indicating a direct role of ERK in arsenic cell transformation. The variability of the specific kinase responses detected in these studies may depend on the specific cell type and concentration of arsenic employed. Activation of different members of MAPK has been related to specific stimuli. For example, ERK is strongly activated by mitogenic stimuli, but only moderately activated by stress.[45] In contrast, both JNK and p38 are activated predominantly by stressors and only moderately by growth factors.[45,46] This argues for the need to conduct in vitro or in vivo studies using the appropriate cell type, i.e., target cells, and at biologically relevant arsenic concentrations.

Arsenic, through activation of transcription factor DNA binding, can modulate not only early-immediate gene expression such as *c-fos, c-jun,* and *c-myc,* whose products are directly involved in cell cycle progression, but also genes of growth factors and cytokines with mitogenic potential. For example, our laboratory has demonstrated that arsenic stimulates tumor growth factor (TGF)α and granulocyte macrophage-colony stimulating factor (GM-CSF) expression in skin of humans exposed to arsenic from drinking water and in human keratinocytes cultures.[22] Overexpression of TGFα, and to a lesser extent GM-CSF, has been associated with neoplastic transformation in the skin,[47,48] and TGFα-transgenic mice exhibit keratinocyte hyperproliferation and tumors in the pancreas, liver, and mammary epithelium,[49] suggesting that TGFα overexpression has the unique ability to complement both tumor initiation and promotion.

We studied the role of arsenic-induced growth factors in mouse skin tumor development in transgenic Tg.AC mice, which carry the v-Ha-*ras* oncogene.[22] Following low-dose application of 12-0-tetradecanoyl phorbol-13-acetate (TPA), a marked increase in the number of skin papillomas occurred in transgenic mice receiving arsenic in drinking water, compared to control mice receiving drinking water without arsenic. Papillomas did not develop in arsenic-treated transgenic mice that had received nontumor-promoting concentrations of TPA or arsenic/ TPA-treated wild-type FVB/N mice. Consistent with earlier in vitro find-

ings, increases in GM-CSF and TGFα mRNA transcripts were found in the epidermis at clinically normal sites following arsenic treatment. Immunohistochemical staining localized TGFα, and GM-CSF overexpression to the hair follicles and injection of neutralizing antibodies to GM-CSF following TPA application reduced the number of papillomas in Tg.AC mice. These results suggest that arsenic enhances development of skin neoplasias via the chronic stimulation of keratinocyte-derived growth factors and may be a rare example of a chemical carcinogen that acts as a copromoter. These findings are supported by a recent observation that rats treated with DMA in their drinking water developed urinary bladder tumors when pretreated with diethylnitrosamine (DEN), a potent chemical initiator.[50]

We employed cDNA microarrays technology to establish the general profile of gene expression induced by arsenite in UROsta cells, a human uroepithelial cell line.[25] These DNA microarrays demonstrated activation of 16 genes at a concentration of 50 μM sodium arsenite, 7 of which were also induced by a concentration of 10 μM arsenite. In addition to previously reported early-immediate genes modulated by arsenic, such as AP-1 and *c-myc*,[20-22] the DNA microarray demonstrated a strong induction of early growth response gene-1 (EGR-1). This gene, which encodes for zinc finger DNA binding transcription factors, has been related to the cell proliferative effects of mitogenic factors such as epidermal growth factor (EGF), nerve growth factor (NGF), or serum.[51] Recently, overexpression of EGR-1 has been associated with human prostate cancer and correlated with the patho-morphological stage of malignancy.[52] Functional EGR-1 binding sites are found in the promoter domains of a large number of genes involved in cell growth, including TGFα, insulin growth factor II (IGF-II), *c-myc,* thymidine kinase, and cyclin D.[52] We also observed that arsenite induced genes implicated in cell growth arrest, such as the growth arrest and DNA damage (GADD) genes GADD153 and GADD45. Increased expression of these genes is associated with effects on endoplasmic reticulum, activation of C/EBP, and modulation of pathways leading to cell death and regeneration.[38] Arsenic also altered genes that encode anti-apoptotic proteins, BCL-2 binding protein and BAG-1, repair associated protein (RAD), and proteins involved in cytoskeleton reorganization.

Taken together, these data suggest that arsenic initiates cell signaling pathways that lead to transcription factors activation such as AP-1 and induction of series of genes involved in regulation of cell metabolic and mitogenic events. It should be cautioned, however, that the precise role of these genes in arsenic-induced malignancies, if any, needs to be defined.

Conclusions

Arsenic is believed to play an important role in development of certain cancers, such as skin and bladder tumors. Although the mechanisms responsible have not been fully defined, tumor promotion through induction of cell signaling pathways that lead to expression of genes involved in cell growth is an intriguing possibility. Identifying the precise pathways through which arsenic affects these cell-cycle-signaling cascades may provide potential targets for therapeutic intervention or prevention of arsenic-related toxicity.

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