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Proposed Mechanisms of Arsenic Toxicity Carcinogenesis

MICHAEL I. LUSTER and PETIA P. SIMEONOVA

National Institute for Occupational Safety and Health, Morgantown, West Virginia

I. INTRODUCTION

Trivalent and pentavalent forms of inorganic arsenic are ubiquitous elements found in nature that unfortunately result in significant human exposure. Oral exposure to arsenic occurs primarily from contamination of drinking water and food constituents, and is particularly high in certain regions of the world including areas of the southwestern United States, eastern Europe, India, China, Taiwan, and Mexico (1,2). Humans can also be exposed to arsenic through inhalation. This occurs primarily in occupations involved in mining/smelting operations, agriculture, or microelectronics (3,4). Epidemiological studies have demonstrated that exposure to inorganic arsenic is associated with increased risk of cancers of the skin and internal organs, including the urinary bladder, respiratory tract, liver, and kidney in populations from Finland, Taiwan, China, Bangladesh, Mexico, southwestern United States, and Central and South America (3–8). Arsenic-induced skin cancers usually develop 20 to 30 years after exposure, and occur in sun-exposed as well as nonexposed areas. The types of skin tumors found include either Bowen's disease, squamous cell carcinomas, basal cell carcinomas, or combined lesions (9–11). The key to identifying patients with arsenic-induced skin tumors is that they normally occur at multiple sites and unusual locations. Internal tumors are also common and are most frequently associated with the bladder. 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 have been identified. Lung tumors from arsenic are often associated with occupational exposure, such as smelters or agriculture workers, and occur from inhalation (12). In addition to neoplasia, additional pathological manifestations of chronic arsenic exposure include skin hyperpigmentation and hyperkera-

tosis (9,13), as well as vascular disease (14,15). Hyperpigmentation is the most common effect in individuals and can occur at any body site, and already pigmented areas are more accentuated. Arsenic-induced hyperpigmentation occurs almost exclusively in individuals of Oriental descent, although the genetic basis for this is not understood (16). Hyperkeratosis, which can appear within 4 years of exposure to arsenic, is manifested primarily in the form of hyperkeratotic papules or plaques and are most commonly found on the palms and soles (17). There are reports of cellular atypia at the base of these papules and on occasion their transformation into basal or squamous cell carcinoma. In contrast to carcinogenicity, little is known regarding the vascular effects of arsenic and most of the reports originate from individuals living in inner Mongolia, Xinjiiang, Toroku, and Nakajo (14,15,18). Circulatory manifestations of arseniasis include increased prevalence of ischemic heart disease and peripheral vascular disease. The latter is commonly known as blackfoot disease in southwestern Taiwan. In addition, dose-response relationships between arsenic exposure and hypertension prevalence have been reported in Southwestern Taiwan (19) and Bangladesh (20).

On the basis of numerous epidemiological studies, arsenic has been classified as a potent 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 (11). It has been estimated that over 350,000 people in the United States 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 (21). Subsequently, there is significant regulatory pressure to lower the acceptable levels. However, epidemiological studies, where exposure levels have been collected, suggest that the current EPA cancer slope factor (CSF) for arsenic may actually overpredict cancer cases at relatively low exposure levels (22). This may be due to the fact that the CSF was calculated assuming a standard linear dose-response relationship while a nonlinear or sublinear dose response may be more appropriate. Human epidemiological data are available providing empirical evidence supporting both a linear (23) and nonlinear (24) association between excess cancer and arsenic exposure. As will be discussed in the following sections, although the precise carcinogenic mechanism for arsenic has not been established, molecular, cellular, and metabolic studies suggest that a nonlinear relation may be most appropriate.

II. ISSUES IN ARSENIC TOXICITY

Although arsenic is classified as a human carcinogen, until recently animal models were considered either negative or equivocal, thus limiting mechanistic studies. We recently established an unconventional animal model in which increased numbers of papillomas develop on the skin of Tg.AC transgenic mice, which contain the human v-Ha ras structural gene, following exposure to arsenite in the drinking water (25). Although this would suggest that arsenic acts as a tumor promoter, it is also necessary to administer low doses of phorbol ester, a classical promoter, to obtain increased papilloma load. This suggests that arsenic acts as a copromoter and is consistent with in vitro studies, implying that arsenic may enhance cell growth rather than activating genotoxic mechanisms. This is also consistent with studies demonstrating that urinary bladder cancers can develop in arsenic-treated rats following exposure to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (26), a potent chemical tumor initiator. However, it has been recently reported that bladder

tumors develop in rats when administered greater than 50 ppm of dimethylarsenic acid (DMA) in the feed for 2 years (27). This is somewhat inconsistent with the current thinking regarding arsenic toxicity, as it suggests that DMA not only acts as a complete carcinogen (i.e., tumor initiator and promoter) but also is more potent than inorganic arsenic. With reference to the latter, the majority of evidence indicates that the inorganic forms of arsenic, particularly iAs^{3+} (arsenite), are more reactive, more toxic, and less readily excreted in the urine than the methylated forms (28). Arsenite and arsenate, in contrast to the methylated forms, have a predilection to react with tissue rich in vicinal dithiols, such as keratins in the skin (29). In mammals, arsenic metabolism first involves the reduction of arsenate (As^{5+}) to arsenite (As^{3+}) by reactions involving glutathione (GSH). Arsenite is then enzymatically methylated, primarily in the liver to monomethylarsonic acid (MMA) and then to DMA, presumably resulting in detoxification and urinary excretion (30). It has been argued that interhuman variability, as well as animal species differences, in arsenic toxicity are due to differences in metabolism. There is even circumstantial evidence in humans that suggests polymorphisms in the methyltransferase enzymes exist which affect efficacy of the oxidative addition of methyl groups to arsenic (31). In addition to the role that methylation plays in arsenic toxicities, another major question, which will be discussed in more detail in the following sections, is the role that reactive oxygen species (ROS) play. It is known that arsenite binds avidly to dithiols, and, when added to cell cultures, has a particular affinity to glutathione resulting in its reduction (32). In this respect, arsenic has been employed historically to elicit heat shock responses, induce oxidant-sensitive enzymes such as heme oxygenase, and stimulate oxidant-sensitive MAP kinase pathways (33–37). The ability to diminish many arsenic-associated cellular responses by addition of *N*-acetylcysteine (NAC) or enhance toxicity by addition of GSH inhibitors, such as L-buthionine sulfoximine, further support these observations. Once GSH is depleted, arsenic either becomes available to interact more freely with so-called "arsenic targets" or there is an increased availability of cell-derived ROS to induce oxidative damage. One current hypothesis that has been actively pursued is that arsenic increases cellular oxidants, which subsequently activate oxidant-sensitive transcription factors, such as AP-1 and NF-κB and affect gene transcription (38–42). It has even been argued that differences observed in cancer rates between populations highly exposed to arsenic may be related to the oxidant levels in their diets (43,44).

III. PROPOSED CLASSICAL GENETIC MECHANISMS OF CARCINOGENICITY

Although there is ample evidence that certain forms of heavy metals, such as cadmium oxide and chromium VI, are carcinogenic because the act as classical genotoxic agents (45,46), this is not supported in studies with arsenic. Arsenic fails to interact directly with DNA to induce mutations, nor is it a DNA-reactive electrophile (47,48). Arsenic causes chromatid abnormalities, such as sister chromatid exchanges, but only at high concentrations that suggest cytotoxicity (45,49). Arsenic induces amplification of the dihydrofolate reductase gene in mouse 3T6 cells and, although gene amplification has been suggested as a possible mechanism of arsenic carcinogenicity (50), it has not been substantiated in other models. DNA repair enzymes are inhibited by arsenic, resulting in a comutagenic response with x-rays, ultraviolet radiation, or alkylating agents (51–53), but epidemiological studies do not support that these other agents are necessary. Since the concentrations

of arsenic required to inhibit DNA ligase activity in vitro are higher than those needed to inhibit repair within cells, it has been argued that arsenic may modulate the control of cellular DNA repair processes (51).

IV. PROPOSED NONCLASSICAL GENETIC MECHANISMS OF CARCINOGENICITY

Considerable studies have focused on the relationship between arsenic metabolism and its ability to alter DNA methylation patterns (54,55). DNA methylation contributes to the control and expression of a number of genes, including protooncogenes such as *c-myc*. Methyltransferase enzymes, which are responsible for the methylation of both DNA as well as arsenic, require cofactors, such as *S*-adenosyl-methionine (SAM) (56). It has been shown that as the level of arsenic exposure in humans increases, the urinary excretion of methylated forms decreases while inorganic arsenic increases correspondingly (57). This is believed to be due either to saturation of methyltransferase, to depletetion of cofactors such as SAM, or to depletion of intracellular GSH. Independent of the cause, the net result would be increased availability of inorganic arsenic that can then react with target tissues. Direct evidence for the hypomethylation hypothesis has been provided in vitro using rat epithelial cells where arsenic-induced cell transformation was found to parallel global DNA hypomethylation in the presence of reduced cellular SAM levels. This was associated with overexpression of the metallothionein gene, a gene controlled by DNA hypomethylation and *c-myc* expression, a marker of cell proliferation (54).

The transcription of some genes is sensitive to hypermethylation, and in apparent contrast to the hypomethylation hypothesis, other studies have suggested that hypermethylation is involved in arsenic carcinogenicity (55). This was first suggested in studies with A549 cells, a type II lung epithelial cell line. When cultured in the presence of arsenic, a dose-responsive cytosine methylation occurs in a portion of the *p53* tumor suppressor gene where transcription is methylation-sensitive. Hypermethylation can be observed within a 341-base pair fragment of the promoter of the cell cycle regulator molecule. Based upon the previously described events involved in arsenic metabolism, the hypermethylation hypothesis cannot be easily explained, but may be due to the existence of methyltransferases with varying sensitivities to arsenic.

V. PROPOSED EPIGENETIC MECHANISMS OF CARCINOGENICITY

As was eluded to earlier, 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 phorbol esters, okadaic acid, and UV radiation, arsenic activates transcription factors, such as AP-1 and AP-2, and induces immediate early genes including *c-fos*, *c-jun*, and *c-myc* (38,58,59), whose products stimulate cell proliferation. Consistent with these observations, arsenic induces a moderate, albeit persistent increase in keratinocyte cell proliferation in vitro as evidenced by increases in thymidine incorporation (25,60), cell cycling (61), labeling of Ki-67, a proliferating cell marker (61), and ornithine decarboxylase activity (62). Recently, it has been demonstrated that fibroblasts (63,64) and human urinary bladder epithelial cells (65) also respond to arsenic in vitro by moderate enhanced cell growth. Electromobility shift assays (EMSAs) and proliferating cell nuclear antigen (PCNA) immunostaining have established that AP-1 activation and hyperplasia can occur concurrently in urinary

bladder epithelial cells and epidermis of mice and rats within 8 weeks following exposure to arsenite (25,65,66). The ability of arsenic to activate AP-1 in vivo has recently been confirmed in transgenic mice which contain an AP-1 luciferase reporter construct (65). Characterization of arsenic-induced AP-1 DNA binding complex has demonstrated that the complex consists of fos/jun heterodimers (58,65), a common heterodimer responsible for regulating cell mitogenesis (67). Of particular relevance to these studies is evidence that *c-jun* expression occurs simultaneously with urinary bladder transitional carcinoma (68,69).

The mechanisms by which arsenic activates these transcription factors may be explained by some of its physicochemical properties. Arsenite accumulates in tissues rich in sulfhydryl-containing molecules such as keratin (70,71), which may explain the accumulation of arsenic in epithelial cells from the skin and bladder and development of carcinogenicity in these tissues. As mentioned earlier, arsenic also reduces intracellular levels of reduced GSH (72). The altered redox state of the cells results in oxidative stress, which can activate oxidant-sensitive transcription factors, such as AP-1 and NF-κB (73). Thus cellular effects of arsenic can be exacerbated by agents, like buthionine-sulfoximine (BSO), which reduce intracellular GSH levels and attenuate in the presence of NAC, a precursor of GSH (74,75). GSH, in addition to serving as an antioxidant, can detoxify and methylate arsenic by direct binding (76). Arsenic-induced GADD 153 expression, a gene whose product is associated with growth arrest and cell damage, although inhibited by NAC, is not affected by 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 direct 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 (77). While this can be due to the generation of ROS, 8-OHdG can also be formed by direct electron transfer without the participation of ROS. Additional studies that measure the ability of arsenic to directly generate ROS 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) (58). It has been demonstrated that arsenite activates *c-jun* N-terminal kinase (JNK) and p38 kinase in HeLa cells in parallel with AP-1 activation and *c-jun*/*c-fos* gene expression. It was suggested that arsenite may interact with sulfhydryl groups on cysteine at the catalytic site of JNK phosphatase to inhibit its activity and prolong JNK and p38 activation. Studies conducted with PC12 cells, used commonly to examine MAPK activation, demonstrated that arsenite is a potent activator of both JNK and p38 kinase, but only moderately activates ERK (40). The activation of all three kinases by arsenic could be prevented by addition of NAC, suggesting a role of GSH and/or oxidative stress in this response. There is evidence that arsenite also activates ERK in PC-12 cells by binding to cysteine-rich domains of the epidermal growth factor receptor (EGFR), which subsequently activates the Ras-dependent pathway (40,78). Previous studies established that arsenic serves as a ligand for receptors that have vicinal thiols in their binding sites, such as glucocorticoid receptors (79), and EGFR would also fall into this category. Recently, it was shown that arsenite activates MAPK in JB6 mouse epidermal cell line, as evidenced by ERK phosphorylation, at doses as low as 0.8 μM while only high doses (>50 μM) activates JNK (41). Furthermore, overexpression of a dominant negative ERK blocked arsenite-induced cell transformation in this cell line, indicating a direct role of ERK in arsenic-induced 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 activated strongly by mitogenic stimuli, but only moderately by stress, while JNK and p38 are strongly activated by stressors and moderately by growth factors (80,81).

Arsenic has been shown to influence 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 that regulate late mitogenic signals including growth factors and certain cytokines. For example, we have demonstrated that transforming growth factor (TGF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression increase in keratinocytes cultured with arsenic as well as in the skin of rodents and humans exposed to arsenic in drinking water (25,82). Immunohistochemical staining has localized TGF-α overexpression to the hair follicles, a site where arsenic tends to concentrate. Overex-

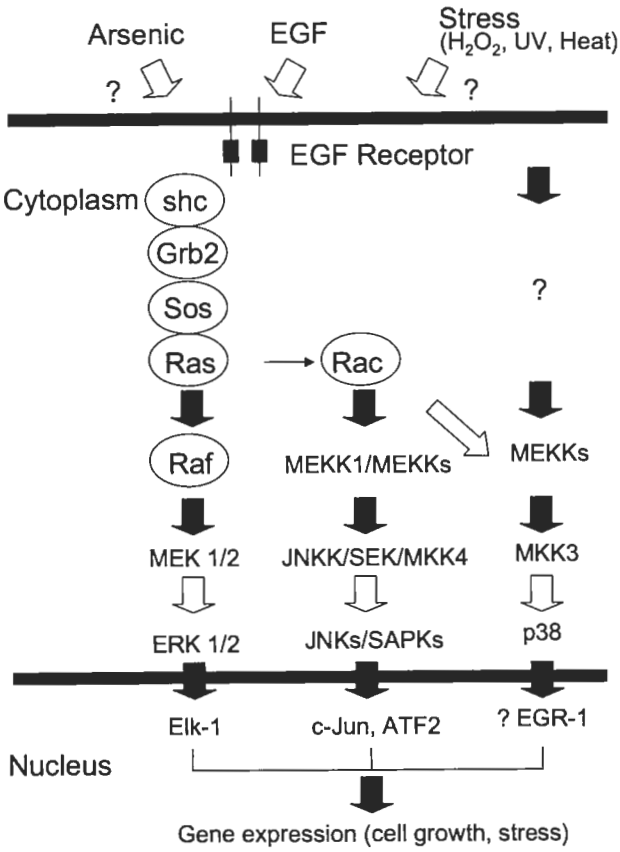

Figure 1 Overview of transduction by MAPKS, ERK1/2, JNKs, SAPKs, and p38. Extracellular signals received by EGF and other stimuli, including possibly arsenic, H_2O_2, UV, or heat, are transduced into the nucleus via the EGF receptor and, subsequently, three cascades that lead to gene expression. Question marks represent unknowns or uncertainties. *Abbreviations*: EGF, epidermal growth factor; ERK extracellular signal-regulated kinase; JNK, Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; SAPK, stress-activated protein kinase; UV, ultraviolet.

pression of TGF-α, and to a lesser extent GM-CSF, has been associated with neoplastic transformation in the skin (83) and TGF-α transgenic mice exhibit hyperkeratoses and increased spontaneous skin and internal tumors (84), suggesting that TGF-α overexpression, like arsenic, has the unique ability to complement both tumor initiation and promotion.

Recently, cDNA microarray technology was employed to establish the profile of gene expression induced by arsenite in UROsta cells, a human uro-epithelial cell line (65). These DNA microarrays demonstrated that arsenic consistently activated 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*, 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 (85). Recently, overexpression of EGR-1 has been associated with human prostate cancer, with its expression correlating with the pathomorphological stage of malignancy. Functional EGR-1 binding sites are found in the promoter domains of a large number of genes involved in cell growth, including TGF-α, insulin growth factor II (IGF-II), *c-myc*, thymidine kinase, and cyclin D (86). Arsenite also activated genes implicated in cellular stress and growth arrest responses, such as GADD153 and GADD45. Activation of these genes is an integral part of endoplasmic reticulum and is associated with activation of C/EBP and modulation of pathways leading to cell death and regeneration (75). Arsenic was also found to alter genes that encode antiapoptotic proteins (BCL-2 binding protein and BAG-1), repair associated protein, and proteins involved in cytoskeleton reorganization. Taken together, these data suggest that arsenic initiates cell signaling pathways that lead to transcription factors, such as AP-1, by binding to EGFR and activating the RAS-dependent pathway or by altering the redox state of the cells, which directly activates nuclear transcription factors that regulate genes involved in stress and mitogenic response (Fig. 1). It should be cautioned, however, that the precise role of these genes in arsenic-induced malignancies is yet to be defined.

VI. ROLE IN CANCER THERAPY

In stark contrast to the carcinogenic effects of chronic arsenic exposure, arsenic trioxide (As_2O_3) has been used therapeutically to successfully treat patients with acute promyelocytic leukemia (APL) without causing severe toxicity (87). In vitro studies have shown that As_2O_3 induces apoptosis at low concentrations in APL cells in contrast to other leukemic cells, which requires considerably higher concentrations (88). The mechanisms responsible for this cell-specific response appear to be associated with the low constitutive levels of GSH peroxidase and higher content of H_2O_2 in APL cells (89). In studies with normal human keratinocytes, it was found that the mitogenic effects of arsenic occur at concentrations only slightly below that which produced cytotoxicity (82). Thus, it would appear that the efficacy of arsenic as a cancer therapy may depend upon the pharmacokinetics of treatment as well as the oxidant status of the target cell.

VII. CONCLUDING REMARKS

Overwhelming epidemiological evidence indicates that arsenic is involved in the development of cancers of the skin and internal organs, as well as certain noncancer toxicities

including dermatotoxicity and vascular disease. Although the mechanisms responsible for its toxicity have not been fully defined, alterations in epigenetic events involving cell growth control appear to be intimately involved. It will be important to identify the precise pathways through which arsenic affects these processes in order to provide potential targets for therapeutic intervention or prevention. The potential need to use nonlinear models in risk assessment dictated either because of unique epigenetic mechanisms or dose-dependent changes in methylation capabilities will provide challenges in conducting accurate low-dose risk assessment. The tissue and species diversity in arsenic metabolism, the role of noncancer pathologies, such as vascular disease, as well as the role of nutrition, will also need to be considered in determining safe levels.

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edited by

JÜRGEN FUCHS
J. W. Goethe University
Frankfurt, Germany

LESTER PACKER
University of Southern California School of Pharmacy
Los Angeles, California

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