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Arsenic toxicity and potential mechanisms of action

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Abstract

Exposure to the metalloid arsenic is a daily occurrence because of its environmental pervasiveness. Arsenic, which is found in several different chemical forms and oxidation states, causes acute and chronic adverse health effects, including cancer. The metabolism of arsenic has an important role in its toxicity. The metabolism involves reduction to a trivalent state and oxidative methylation to a pentavalent state. The trivalent arsenicals, including those methylated, have more potent toxic properties than the pentavalent arsenicals. The exact mechanism of the action of arsenic is not known, but several hypotheses have been proposed. At a biochemical level, inorganic arsenic in the pentavalent state may replace phosphate in several reactions. In the trivalent state, inorganic and organic (methylated) arsenic may react with critical thiols in proteins and inhibit their activity. Regarding cancer, potential mechanisms include genotoxicity, altered DNA methylation, oxidative stress, altered cell proliferation, co-carcinogenesis, and tumor promotion. A better understanding of the mechanism(s) of action of arsenic will make a more confident determination of the risks associated with exposure to this chemical.

Keywords: Arsenic; Toxicity; Mechanisms

Arsenic is a metalloid found in water, soil, and air from natural and anthropogenic sources. It exists in inorganic and organic forms and in different oxidation states (-3, 0, $+3$, $+5$). In the case of environmental exposure, toxicologists are primarily concerned with arsenic in the trivalent and pentavalent oxidation state. Fig. 1 displays the structure of several arsenicals of toxicological relevance. The more commonly known arsenic compounds, arsenate and arsenite, are the anionic forms of arsenic acid and arsenous acid, respectively. Monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) are stable methylated mammalian metabolites of inorganic arsenic and are primarily excreted in the urine. An item of interest is that DMA^V and the sodium salts of MMA^V have been used as herbicides.

1. Metabolism of arsenic

The metabolism of arsenic has an important role in its toxic effects. Many, but not all, mammalian species methylate inorganic arsenic (Vahter, 1994). There is also variation between species and among human populations in the rate and extent of methylation of inorganic arsenic

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(Vahter, 1999, 2000). Inorganic arsenic is metabolized by a sequential process involving a two-electron reduction of pentavalent arsenic to trivalent arsenic, followed by oxidative methylation to pentavalent organic arsenic (reviewed in Thomas et al., 2001). The reduction can occur nonenzymatically in the presence of a thiol such as glutathione (GSH) (Delnomdedieu et al., 1994b; Scott et al., 1993). However, human liver arsenate (Radabaugh and Aposhian, 2000) and MMA^V (Zakharyan et al. 2001) reductases have been partially purified and the latter enzyme appears to be a glutathione-*S*-transferase (omega). The methylation of arsenic is enzymatic, requiring *S*-adenosylmethionine (SAM) and a methyltransferase.

The predominant metabolite of inorganic arsenic, dimethylarsinic acid ($(CH_3)_2As^VO(OH)$), is rapidly excreted by most mammals. Trimethylarsine oxide (TMAO, $(CH_3)_3As^VO$)) is the final product in this scheme, but is found in very low amounts in urine, if at all, after exposure to inorganic arsenic. For many years, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) have been proposed intermediates in the metabolism of arsenic. Recently, MMA^{III} and DMA^{III} have been detected in the urine of humans chronically exposed to inorganic arsenic in their drinking water (Aposhian et al., 2000; Del Razo et al., 2001b) and in the bile of rats administered arsenite intravenously (Gregus et al., 2000).

$$As^VO(OH)_3 + 2e^- \rightarrow As^{III}(OH)_3 + CH_3^+ \rightarrow CH_3As^VO(OH)_2 + 2e^- \rightarrow CH_3As^{III}(OH)_2 + CH_3^+$$
$$\rightarrow (CH_3)_2As^VO(OH) + 2e^- \rightarrow (CH_3)_2As^{III}OH + CH_3^+ \rightarrow (CH_3)_3As^VO$$

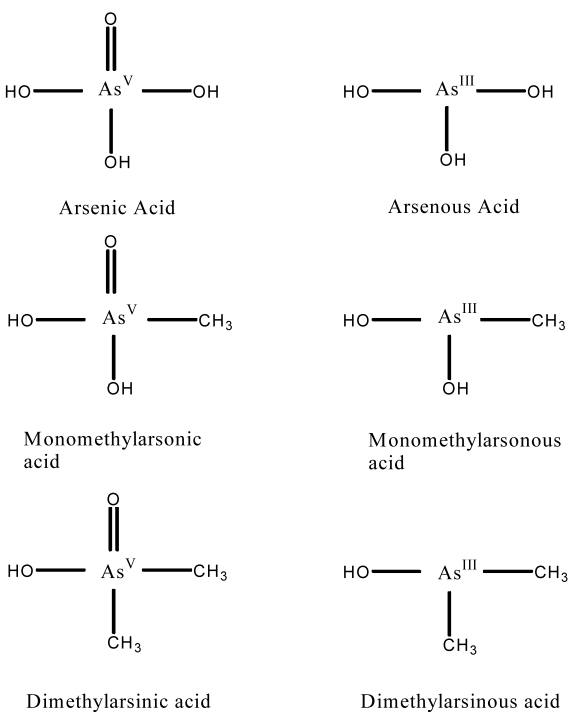

Fig. 1. Structures of some toxicologically relevant arsenic compounds.

2. Acute toxicity

The acute toxicity of arsenic is related to its chemical form and oxidation state. The LD_{50} values of several arsenicals in laboratory animals are displayed in Table 1. A basic tenet is that the acute toxicity of trivalent arsenic is greater than pentavalent arsenic. For example, in the mouse, the oral LD_{50} of arsenic trioxide is more than 36-fold lower than that of MMA^V. In the human adult, the lethal range of inorganic arsenic is estimated at a dose of 1–3 mg As/kg (Ellenhorn, 1997). The characteristics of severe acute arsenic toxicity in humans include gastrointestinal discomfort, vomiting, diarrhea, bloody urine, anuria, shock, convulsions, coma, and death.

For many years it was believed that the acute toxicity of inorganic arsenic was greater than organic arsenic and hence, the methylation of inorganic arsenic was a detoxication reaction. This dogma was held because DMA^V, the primary excreted metabolite of inorganic arsenic, is less acutely toxic than inorganic arsenic. However, Cullen et al. (1989) found that a derivative of MMA^{III} is more toxic than arsenite to the mi-

Table 1
Acute toxicity of arsenic in laboratory animals

Chemical	Species (sex)	Route	LD_{50} (mg As/kg)	Reference
Arsenic trioxide[a]	Mouse (m)	Oral	26	Kaise et al. (1985)
Arsenic trioxide	Mouse (m)	Oral	26–48[b]	Harrison et al. (1958)
Arsenic trioxide	Rat (m/f)	Oral	15	Harrison et al. (1958)
Arsenite	Mouse (m)	im	8	Bencko et al. (1978)
Arsenite	Hamster (m)	ip	8	Petrick et al. (2001)
Arsenate	Mouse (m)	im	22	Bencko et al. (1978)
MMA^{III}	Hamster (m)	ip	2	Petrick et al. (2001)
MMA^{V}	Mouse (m)	Oral	916	Kaise et al. (1989)
DMA^{V}	Mouse (m)	Oral	648	Kaise et al. (1989)
$TMAO^{V}$	Mouse (m)	Oral	5500	Kaise et al. (1989)
Arsenobetaine[c]	Mouse (m)	Oral	>4260	Kaise et al. (1985)

[a] Arsenic trioxide, As_2O_3 is a trivalent arsenical.
[b] Four strains of mice were treated.
[c] Pentavalent organic arsenic.

croorganism *Candida humicola* in vitro. Human cells are also more sensitive to the cytotoxic effects of MMA^{III} than arsenite (Petrick et al., 2000; Styblo et al., 1999, 2000). DMA^{III} is at least as cytotoxic as arsenite in several human cell types (Styblo et al., 2000). Recently Petrick et al. (2001) reported that MMA^{III} has a lower LD_{50} than arsenite in the hamster. The greater acute toxicity of the methylated trivalent intermediates of arsenic suggests that the methylation of arsenic is not solely a detoxication mechanism.

3. Chronic toxicity

Many different systems within the body are affected by chronic exposure to inorganic arsenic. Some of these systems and their associated toxic effects from chronic arsenic exposure are listed in Table 2. One of the hallmarks of chronic toxicity in humans from oral exposure to arsenic are skin lesions, which are characterized by hyperpigmentation, hyperkeratosis, and hypopigmentation (Yeh et al. 1968; Cebrian et al., 1983). In Taiwan, Blackfoot disease, a vasoocclusive disease which leads to gangrene of the extremities, is also observed in individuals chronically exposed to arsenic in their drinking water (Tseng, 1977).

4. Mechanism of pentavalent arsenic toxicity

Arsenate can replace phosphate in many biochemical reactions because they have similar structure and properties (Dixon, 1997). For example, arsenate reacts in vitro with glucose and gluconate (Lagunas, 1980; Gresser, 1981) to form glucose-6-arsenate and 6-arsenogluconate, respectively. These compounds resemble glucose-6-phosphate and 6-phosphogluconate, respectively. Glucose-6-arsenate is a substrate for glucose-6-phosphate dehydrogenase and can inhibit hexokinase, as does glucose-6-phosphate (Lagunas,

Table 2
Effects observed in humans and laboratory animals after chronic arsenic exposure

System	Effect
Skin	Skin lesions
Cardiovascular	Blackfoot disease
Nervous	Peripheral neuropathy, encephalopathy
Hepatic	Hepatomegaly, cirrhosis, altered heme metabolism
Hematological	Bone marrow depression
Endocrine	Diabetes
Renal	Proximal tubule degeneration, papillary and cortical necrosis

1980). Arsenate can also replace phosphate in the sodium pump and the anion exchange transport system of the human red blood cell (Kenney and Kaplan, 1988). When KB oral epidermoid carcinoma cells are grown in phosphate-free media, arsenate accumulates in the cells at a greater rate (Huang and Lee, 1996). Inhibition of arsenate uptake by phosphate in these cells is dose-dependent.

Arsenate uncouples in vitro formation of adenosine-5′-triphosphate (ATP) by a mechanism termed arsenolysis. At the substrate level, arsenolysis may occur during glycolysis. In one step of the glycolytic pathway, phosphate is linked enzymatically to D-glyceraldehyde-3-phosphate to form 1,3-biphospho-D-glycerate. Arsenate can replace phosphate in this reaction to form the anhydride 1-arsenato-3-phospho-D-glycerate. However, this anhydride is unstable and it hydrolyzes to arsenate and 3-phosphoglycerate. The lower stability of the arsenic anhydride may be due to the As–O bond length, which is approximately 10% longer than the P–O bond length (Dixon, 1997). ATP is generated during glycolysis in the presence of phosphate (substrate level phosphorylation), but not arsenate (Crane and Lipmann, 1953; Aposhian, 1989). At the mitochondrial level, arsenolysis may occur during oxidative phosphorylation. Adenosine-5′-diphosphate–arsenate is synthesized by submitochondrial particles from adenosine-5′-diphosphate (ADP) and arsenate in the presence of succinate (Gresser, 1981). ADP–arsenate hydrolyzes easily compared to ADP–phosphate (ATP), which is formed during oxidative phosphorylation. At both the substrate and mitochondrial level, arsenolysis diminishes in vitro formation of ATP by the replacement of phosphate with arsenate in the enzymatic reactions.

Depletion of ATP by arsenate has been observed in cellular systems. ATP levels are reduced in rabbit (Delnomdedieu et al., 1994a) and human erythrocytes (Winski and Carter, 1998) after in vitro exposure to arsenate (rabbit: 0.8 mM; human: 0.01–10 mM). Arsenite is ineffective in epleting ATP in human erythrocytes.

5. Mechanism of trivalent arsenic toxicity

Specific functional groups within enzymes, receptors or coenzymes, such as thiols or vicinal sulfhydryls, have a major role in the activity of these molecules. Trivalent arsenicals readily react in vitro with thiol-containing molecules such as GSH and cysteine (Scott et al., 1993; Delnomdedieu et al., 1994b). Binding of MMA^{III} and DMA^{III} to protein in vitro occurs to a greater extent than with the pentavalent organic forms (Styblo et al., 1995). Arsenite has a higher affinity for dithiols than monothiols, as shown by the highly favored transfer of arsenite from a $(GSH)_3$-arsenic complex to the dithiol 2,3-dimercaptosuccinic acid (Delnomdedieu et al., 1993). The binding of trivalent arsenic to critical thiol groups may inhibit important biochemical events which could lead to toxicity. However, binding of arsenite at nonessential sites in proteins may be a detoxication mechanism (Aposhian, 1989).

Pyruvate dehydrogenase (PDH) is a multi subunit complex that requires the cofactor lipoic acid, a dithiol, for enzymatic activity. Arsenite inhibits PDH (Peters, 1955; Szinicz and Forth, 1988; Hu et al., 1998), perhaps by binding to the lipoic acid moiety. Petrick et al. (2001) has shown that MMA^{III} is a more potent inhibitor of PDH than arsenite. PDH oxidizes pyruvate to acetyl–CoA, a precursor to intermediates of the citric acid cycle. The citric acid cycle degrades the intermediates, and this provides reducing equivalents to the electron transport system for ATP production. Inhibition of PDH may ultimately lead to decreased production of ATP. Also, intermediates of the citric acid cycle can be used in gluconeogenesis. Inhibition of PDH may explain in part the depletion of carbohydrates observed in rats administered arsenite (Szinicz and Forth, 1988; Reichl et al., 1988).

Methylated trivalent arsenicals such as MMA^{III} are potent inhibitors of GSH reductase (Styblo et al., 1997) and thioredoxin reductase (Lin et al., 1999). The inhibition may be due to the interaction of trivalent arsenic with critical thiol groups in these molecules. The activity of the methylated trivalent arsenicals is greater than arsenite, MMA^{V}, and DMA^{V}. Inhibition of these enzymes

may alter cellular redox status and eventually lead to cytotoxicity.

6. As carcinogenicity

Inorganic arsenic is classified by the International Agency for Research on Cancer (IARC, 1980, 1987) and the US Environmental Protection Agency (EPA, 1988) as a known human carcinogen. This classification is based on several epidemiological studies which show an association of exposure to arsenic and the development of cancer. Cancer has developed in individuals exposed to arsenic through medical treatment with Fowler's solution (potassium arsenite) (Fierz, 1965), occupational exposure via inhalation at copper smelters (Lee-Feldstein, 1983, 1986) or naturally contaminated drinking water (Tseng et al., 1968; Cebrian et al., 1983). Tumors that develop after inhalation of arsenic are observed primarily in the lung (Lee-Feldstein, 1983, 1986), whereas they are initially observed in the skin after oral exposure to arsenic (Tseng et al., 1968; Cebrian et al., 1983). However, additional studies indicate that cancer of internal organs occurs in individuals who chronically consume arsenic-contaminated drinking water. Tumor sites include bladder, liver, and kidney (Smith et al., 1992; Bates et al., 1995).

Although there is sufficient evidence to classify arsenic as a human carcinogen, whether it occurs in laboratory animals is not clear. In several studies, a carcinogenic effect was absent in animals after a life-time exposure to inorganic arsenic in either the drinking water (arsenite, 5 ppm As) or diet (arsenite, 5–125 ppm As; arsenate, 5–250 ppm As), or after oral intubation (0.1 mg/kg arsenate, 5 days/week). This has been reported in rats (Schroeder et al. 1968; Kanisawa and Schroeder, 1969; Byron et al., 1967), dogs (Byron et al., 1967), and monkeys (Thorgeirsson et al., 1994). Mice exposed to arsenite (10 ppm) for 26 weeks in their drinking water also did not develop tumors (Rossman et al., 2001).

However, there have been several positive reports on the carcinogenicity of arsenic in animal models. In the 1980s, several studies (Ishinishi et al., 1983; Pershagen et al., 1984; Pershagen and Bjorklund, 1985; Yamamoto et al., 1987) reported on the carcinogenicity of inorganic arsenic in hamsters after intratracheal instillation. This route was used to simulate inhalation of arsenic. Chemicals used in these studies included arsenic trioxide (As_2O_3), calcium arsenate ($Ca(AsO_4)_2$), and arsenic trisulfide (As_2S_3). The animals were dosed (total dose: 3.75 or 5.25 mg As) one time per week for 15 weeks and monitored for tumors throughout their life span. There was greater mortality with As_2O_3 compared to $Ca(AsO_4)_2$ and As_2S_3, and this effect was dose-dependent. Up to 50% of the animals in the As_2O_3-treated groups did not survive the dosing regimen, and this result must be considered in light of the tumorigenic response. After exposure to As_2O_3, adenocarcinomas, carcinomas, and adenomas developed in the respiratory tract. After exposure to $Ca(AsO_4)_2$, carcinomas and adenomas were observed and there were generally more tumors found than with As_2O_3. The effect of $Ca(AsO_4)_2$ may be due to its longer retention in the lung than the other arsenicals (Pershagen et al., 1982). There was only a minimal tumorigenic effect in the animals administered As_2S_3.

Germolec et al. (1997) used a transgenic mouse strain (TG.AC), which has genetically initiated skin (from a mutated v-Ha-*ras* oncogene), to examine arsenic carcinogenicity. Mice were exposed to sodium arsenite (200 ppm) in drinking water for 4 weeks, followed by application of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on shaved skin, two times per week for 2 weeks. Six weeks after exposure to TPA, there was a 4-fold greater incidence of skin papillomas in arsenic-exposed, TPA-treated mice compared to mice treated only with TPA. Papillomas were not observed in control or mice exposed only to arsenite.

Wei et al. (1999) reported that DMA^V, the predominant metabolite of inorganic arsenic, is a rat bladder carcinogen. Male F344 rats were administered DMA^V (0, 12.5, 50, and 200 ppm) in drinking water for 2 years. The treatment had no effect on survival rates between experimental groups and bladder tumors were not observed in the control group. The incidence of bladder tu-

mors was dose-dependent (12.5 ppm: 0/33 rats; 50 ppm: 8/31 rats; 200 ppm: 12/31). Treatment did not affect urinary pH and bladder calculi were not observed in these animals. However, the doses used in this study must be carefully viewed, because it is unlikely that levels this high would occur in vivo after exposure to inorganic arsenic at nonlethal doses. Also, the rat is unique in that its red blood cells accumulate and retain DMA (Odanka et al., 1980; Vahter et al., 1984). The pharmacokinetics of arsenic is different in the rat than other species, and this effect may also have some role in the carcinogenic effect of DMA^V.

Ng et al. (1999), as described in a conference report, showed that multiple tumors develop in mice exposed to arsenic in drinking water. This group exposed C57BL/6J mice and metallothionein knockout (MT^-) mice to sodium arsenate (500 μg As/L) in drinking water up to 26 months. In both strains, tumors were found in the gastrointestinal tract, lung, liver, spleen, bone, skin, reproductive system, and eye. The percentage of tumor-bearing animals was higher in the C57BL/6J (41%) mice than in the MT^- (26%) mice, which suggests that metallothionein is not protective of the development of arsenic-induced tumors. Tumors were not observed in control animals, which were given tap water containing < 0.1 μg As/L. This strain of mice apparently has a very low incidence of spontaneous tumors (Hoag, 1961; Adkinson and Sundberg, 1991).

K6/ODC transgenic mice exposed to arsenite (10 ppm) or DMA^V (10 and 100 ppm) for 20 weeks develop a low, but significant number of skin tumors (Chen, 2000). The skin of this transgenic mouse overexpresses ornithine decarboxylase (ODC), an enzyme involved in polyamine biosynthesis and an indicator of cell proliferation. Tumor promotion in mouse skin can occur under conditions of overexpression of ODC (O'Brien et al., 1997).

7. Mechanisms of action for arsenic carcinogenicity

Although it is clear that arsenic is a human carcinogen, and there are some positive results in animal studies, the mechanism of action is unknown. Many different mechanisms of action have been proposed and several have recently been evaluated by experts in the field (ERG, 1997; NRC, 1999, 2001). Some proposed mechanisms include genotoxicity, cell proliferation, altered DNA repair and DNA methylated oxidative stress, co-carcinogenesis, and tumor promotion. Defining a mechanism of action for arsenic carcinogenicity has been difficult for several reasons. Some of these include the several negative results of a carcinogenic effect of inorganic arsenic in a standard animal bioassay, the lack of evidence that arsenic is a point mutagen, the carcinogenic and promoting effects of DMA^V, the toxic effects of the trivalent methylated forms of arsenic, and the myriad effects of arsenic on cell signaling.

7.1. Genotoxicity

7.1.1. In vitro genotoxicity

Inorganic arsenic does not appear to be a point mutagen in standard assays. Sodium arsenite is not mutagenic in tests selecting for *Escherichia coli* tryptophan$^+$ revertants or Chinese hamster ovary cells when selecting for ouabain- (ATPase) or thioguanine-resistant (hypoxanthine phosphoribosyl transferase, HPRT) mutants (Rossman et al., 1980). Arsenite does, however, induce large deletion (multilocus) mutations in hamster–human hybrid cells (Hei et al., 1998).

Chromosomal aberrations, DNA–protein crosslinks, and sister chromatid exchanges are observed in hamster embryo cells (Rossman et al., 1980; Lee et al., 1985b; Kochhar et al., 1996), and human lymphocytes (Larramendy et al., 1981; Jha et al., 1992; Wiencke and Yager, 1991; Rasmussen and Menzel, 1997) and fibroblasts (Okui and Fujiwara, 1986; Jha et al., 1992; Dong and Luo, 1993) after exposure to inorganic arsenic. The chromosomal aberrations are characterized by chromatid gaps, breaks and fragmentation, endoreduplication, and chromosomal breaks. These effects are dose-dependent and arsenite is more potent than arsenate.

The pentavalent organic arsenicals are genotoxic in Chinese hamster lung (V79) cells (Endo et al., 1992; Eguchi et al., 1997) and human

lung cells (Tezuka et al., 1993; Yamanaka et al., 1995), but require much greater doses than the inorganic arsenicals. The effects include excess tetraploidy (DMA^V, $TMAO^V$), mitotic arrest (MMA^V, DMA^V, and $TMAO^V$), DNA single strand scissions (DMA^V), and DNA–protein cross links (DMA^V).

A recent and important study by Mass et al. (2001) has shown that the trivalent methylated arsenicals, MMA^{III} and DMA^{III} are directly genotoxic. Supercoiled ϕX174 DNA was nicked by DMA^{III} (150 μM) and MMA^{III} (30 mM). Neither arsenite, arsenate, MMA^V, nor DMA^V were able to nick the supercoiled DNA. DMA^{III} and MMA^{III} also damaged human lymphocyte DNA, and were 386 and 77 times more potent, respectively, than arsenite.

7.1.2. Co-mutagenesis of inorganic arsenic

Inorganic arsenic (As_2O_3, arsenite, and arsenate) is co-mutagenic with other chemicals and ultraviolet (UV) light in mammalian cells. A synergistic increase in UV-induced chromatid and chromosomal aberrations and mutation at the HPRT locus in Chinese hamster ovary cells is observed after the UV-treated cells are exposed to inorganic arsenic (Lee et al., 1985a; Okui and Fujiwara, 1986; Li and Rossman, 1991). There is no co-mutagenic effect of arsenic with UV-induced sister chromatid exchanges or ouabain resistance (Lee et al., 1985a).

As observed in animal cells, arsenic is co-mutagenic with chemicals and electromagnetic radiation in human cells. Chromosomal aberrations induced by the DNA crosslinking agent diepoxybutane in lymphocytes (Wiencke and Yager, 1991) and X-rays or UV light in fibroblasts (Jha et al., 1992) are potentiated after exposure to sodium arsenite. There is no synergistic effect of arsenic on sister chromatid exchanges induced by diepoxybutane or UV light.

7.1.3. In vivo genotoxicity

Administration of arsenite to mice results in a linear dose-dependent increase in micronucleated polychromatic erythrocytes (Deknudt et al., 1986; Tinwell et al., 1991). Micronuclei induced by arsenite are observed only in somatic cells. Also observed in bone marrow cells of mice administered arsenite are chromosomal aberrations including chromatid gaps and breaks, and chromosomal rearrangements (Das et al., 1993; Roy-Choudhury et al., 1996). Crude garlic extract administered before exposure to arsenite reduces its clastogenic effect (Das et al., 1993; Roy-Choudhury et al., 1996). Garlic extract is composed of several sulfur-containing chemicals which may interact with trivalent arsenic and inhibit its toxic effect.

The hepatic methyl donor status is a factor that may affect arsenic-induced genotoxicity (Tice et al., 1997). Mice fed a choline-deficient diet have a lower hepatic methyl donor status than mice fed a choline-sufficient diet. In choline-deficient mice, urinary excretion of inorganic and organic arsenic is decreased after exposure to inorganic arsenic. After four daily oral doses of sodium arsenite, a significant increase in frequency of micronucleated erythrocytes at 24 h is observed in choline-sufficient and -deficient mice. No effect is observed after an acute dose. Choline sufficient arsenite-exposed mice have greater bone marrow toxicity, exhibited as a significant decrease in % polychromatic erythrocytes within the total erythrocyte population, compared to choline deficient arsenite exposed mice. DNA crosslinking is increased in bladder and liver parenchymal cells of choline-sufficient mice after a single or multiple exposures to arsenite. In contrast, increased DNA crosslinking is observed in skin of choline-deficient mice after single or multiple exposures to arsenite. Arsenite-induced genotoxicity shifts from liver and bladder to skin when the hepatic methyl status of the mouse is deficient.

After exposure to DMA^V (1.5 g/kg), DNA single strand scissions and DNA-protein crosslinks are observed in mouse and rat lung (Yamanaka et al., 1989a,b, 1991; Brown et al., 1997). The damage is not observed in the mouse until 12 h after exposure and is repaired by 24 h. DNA single strand scissions are not observed in mouse or rat liver (Yamanaka et al., 1989b; Brown et al., 1997) or mouse kidney (Yamanaka et al., 1989b) after exposure to DMA^V.

7.1.4. Gene amplification

Mouse 3T6 cells grown in media containing inorganic arsenic become resistant to the toxic effects of methotrexate (Lee et al., 1988). The number of resistant cells is dependent on the dose of arsenic, and arsenite is more potent than arsenate. Arsenic enhances the amplification of the gene which codes for the enzyme dihydrofolate reductase. Lee et al. (1988) suggested that the gene amplification induced by arsenic may have a role in its carcinogenic effect. This proposal is based on the observations that arsenic is not a point mutagen, yet is carcinogenic, and that oncogenes are amplified in several animal and human tumors.

7.1.5. Transformation

Sodium arsenate and sodium arsenite induce a dose-dependent transformation of Syrian hamster embryo cells (Lee et al., 1985b) and BALB/3T3 cells (Bertolero et al., 1987; Saffioti and Bertolero, 1989). Arsenite is 4–10-fold more potent than arsenate in inducing transformation, due in part to the greater cellular uptake of arsenite (Bertolero et al., 1987). In hamster cells, neither arsenate nor arsenite were mutagenic at either the ATPase or HPRT genetic loci at doses which induced transformation (Lee et al., 1985b). Cytogenetic analysis of the transformed cells showed endoreduplication, chromosomal aberrations, and sister chromatid exchanges. BALB/3T3 cells transformed by arsenite were administered to nude mice subcutaneously and tumors developed (Saffioti and Bertolero, 1989). The tumors grew rapidly and appeared as fibrosarcomas, but did not metastasize to other sites within the mice.

The rat liver cell line TRL 1215 is transformed by arsenite (Zhao et al., 1997). Cells were transformed after exposure of 8 weeks or more to arsenite and then were inoculated into athymic nude mice. There was a dose-dependent development of tumors at the site of administration, and the tumors metastasized to the lungs. The cells retained their state of transformation and ability to induce tumors in mice, even after removal of arsenite from the culture media, suggesting that a permanent genetic change occurred.

7.2. Altered DNA repair

Inhibition of DNA repair may ultimately lead to a genotoxic event. DNA repair is inhibited in arsenic-treated cells, and this inhibition may result in a co-mutagenic effect with X-rays, UV light, and several chemicals. DNA excision repair of thymine dimers in human fibroblasts is inhibited by inorganic arsenic, with As_2O_3 being more potent than sodium arsenate (Okui and Fujiwara, 1986). DNA ligase activity in nuclear extracts is decreased by arsenite (55% decrease at 10 μM arsenite) (Li and Rossman, 1989), but the enzyme is not directly inhibited (Li and Rossman, 1989; Hu et al., 1998). Hu et al. (1998) suggest that arsenite may indirectly inhibit DNA ligase activity by altering cellular redox levels or affecting signal transduction pathways and phosphorylation of proteins which are linked to DNA ligase activity.

Eukaryotic cells respond to DNA strand breaks by stimulating the enzyme poly-(ADP-ribose)polymerase, and this enzyme may have a role in DNA repair (Berg, 1985; Grube et al., 1991). Yager and Wiencke (1997) observed a significant dose-dependent decrease in activity of this enzyme in human T-cell lymphoma cells. At a dose of 10 μM arsenite, there is approximately a 50% decrease in enzyme activity and 80% cell viability. The enzyme contains two vicinal sulfhydryl groups, and arsenite may bind one or both groups and inhibit the enzyme.

7.3. Altered DNA methylation

Altered DNA methylation may have a prominent role in the development of cancer (Counts and Goodman, 1995). Two studies suggest that alteration of DNA methylation by arsenic may have a role in its carcinogenicity. Mass and Wang (1997) examined the effect of arsenic on DNA methylation patterns of the tumor suppressor gene *p53* in the human adenocarcinoma cell line A549. Arsenite increased the resistance of the *p53* promoter region to cleavage by the restriction enzyme *Hpa*II, which cleaves unmethylated cytosine within the CCGG sequence. The increased resistance to cleavage indicates more methylated

cytosine was present within the *p53* gene. The ability of transcription factors to bind DNA, which would modify gene expression, may be altered by methylated cytosine. Arsenate was less potent in increasing the methylation of cytosine, and DMA^V was ineffective at the doses used in this study. The hypermethylation induced by arsenite was confirmed by DNA sequencing of the promoter region and using bisulfite to visualize 5-methylcytosine. It appears that the sequence CpG within the entire genome was methylated, because of diminished ability of *Sss*I methylase to transfer methyl groups from SAM to DNA. Although the *p53* gene was hypermethylated, it is not known if the expression of it was altered.

The DNA of rat liver TRL 1215 cells transformed by arsenite is globally hypomethylated and the effect is dependent on dose and length of exposure (Zhao et al., 1997). DNA hypomethylation might result in aberrant gene expression, and hence transformation of the cells. The DNA of cells treated acutely with arsenite or at doses that did not transform them is not hypomethylated. In the transformed cells, the levels of the methyl donor SAM is decreased. Although the levels of *S*-adenosylhomocysteine (SAH), the product formed from SAM after the methyl group is transferred, are unaffected, the SAM/SAH ratio is significantly decreased. Activity of DNA methyltransferase in the arsenic-transformed cells is decreased by up to 40%, but it is not affected by an acute exposure to arsenite. Expression of the DNA methyltransferase gene increased two-fold in the transformed cells, which was attributed to an attempt by the cells to increase methyltransferase activity.

While the results from the two studies described above may appear to be contrasting, Zhong and Mass (2001) observed both hypomethylation and hypermethylation of DNA in human lung cells after exposure to arsenite for several weeks. Although the effect of the altered DNA methylation in the cells was not determined, the results indicate that both states (hypo- and hypermethylation) can exist in arsenite-treated cells. This suggests that altered methylation within a specific DNA sequence may be more important than the absolute level of DNA methylation.

7.4. Oxidative stress

Oxidative stress arises when reactive oxygen species are generated that can react with cellular constituents such as thiols and lipids. Depletion of GSH by oxidants, for example, may alter the redox status of the cell and present a stressful and toxic situation. Arsenic appears to induce oxidative stress both in vitro and in vivo. Arsenic induces heat shock or stress proteins in cultured human cells (Keyse and Tyrell, 1989) and in vivo (Brown and Rush, 1984) (reviewed by Del Razo et al., 2001a). Reactive oxygen species are detected in human-hamster hybrid cells within 5 min after exposure to arsenite (Liu et al., 2001). Catalase and superoxide dismutase, enzymes which metabolize oxidants, reduce arsenite-induced micronuclei in Chinese hamster ovary cells (Wang and Huang, 1994) and sister chromatid exchanges in human lymphocytes (Nordenson and Beckman, 1991). Antioxidants such as vitamin E, methylamine and benzyl alcohol reduce the killing of human fibroblasts by arsenite (Lee and Ho, 1994). A DMA peroxyl radical has been detected in vitro (Yamanaka et al., 1990) and oxidative damage in the lungs of mice is observed after exposure to DMA^V (Yamanaka et al., 1991).

Trivalent organic arsenicals inhibit GSH reductase (Styblo et al., 1997) and thioredoxin reductase (Lin et al., 1999). Inhibition of these enzymes may result in a decreased ability of cells to protect against oxidants. Gene expression is also affected by levels of thioredoxin, because it has a role in the regulation of the binding of transcription factors to DNA (Arrigo, 1999; Powis et al., 2000).

Reactive oxygen species that damage DNA in vitro are generated from iron released from ferritin. In the presence of ascorbic acid, DMA^{III} and DMA^V significantly enhanced iron release from ferritin (Ahmad et al., 2000). In the absence of ascorbic acid, only DMA^{III} was active in the release of iron. Arsenate, arsenite, MMA^V and MMA^{III} were inactive in the release of iron.

7.5. Cell proliferation

The development of cancer involves in part the uncontrolled proliferation of cells. Cell prolifera-

tion can result from cytotoxicity and consequent regeneration of the cells and/or direct mitogenesis (Cohen and Ellwein, 1990). Increased cell proliferation is observed in rat bladder (Wanibuchi et al., 1996) after an 8-week exposure to DMA^V (10–100 ppm) in drinking water and rat kidney (Murai et al., 1993) after 4-week oral administration of DMA^V (57–113 mg/kg/day, 5 days/week). Microscopic examination of the urinary bladder of mice treated with DMA^V for 8 weeks showed increased alteration of epithelial cell surfaces and labeling of cells with 5-bromo-2′-deoxyuridine. There was a lower labeling index in the higher DMA^V dose group, which was suggested to result from the higher toxicity of the DMA^V in this group. Urothelial cell proliferation was observed in rats fed DMA^V in the diet (40 and 100 ppm) (Arnold et al., 1999). The effects were reversible after removing DMA^V from the diet. Rat bladder carcinogenesis in DMA^V exposed rats appears to be related to toxicity and regeneration of the urothelium (Arnold et al., 1999).

Induction of ODC, an indicator of cellular proliferation, is observed in rat liver after administration of arsenite (Brown and Kitchin, 1996).

The skin of transgenic (TG.AC) mice administered arsenite in drinking water show epidermal thickening and hyperkeratosis (Germolec et al., 1997). Human keratinocytes proliferate after exposure to arsenite in culture (Germolec et al., 1996), and growth factors within these cells, as well as the skin of transgenic mice, are stimulated (Germolec et al., 1997). At low doses (1–10 nM), arsenite, MMA^{III}, and DMA^{III} stimulate normal human epidermal keratinocyte proliferation (Vega et al., 2001). In contrast, arsenate, MMA^V, and DMA^V did not stimulate cellular proliferation. The secretion of growth promoting cytokines by the keratinocytes was also significantly elevated by the three trivalent arsenicals.

Multiple genes and proteins involved in proliferative signaling and mitogenesis in murine fibroblasts are affected after an 8-week exposure to arsenite (Trouba et al., 2000a). After stimulation by epidermal growth factor, a greater proportion of arsenite-exposed fibroblasts enter the S-phase of the cell cycle than control cells. The arsenite-exposed cells also exhibit an increased expression of positive regulators of proliferation (*c-myc* and E2F-1) and a decreased expression of negative regulators of proliferation (MAP kinase phosphatase-1 and $p27^{kip1}$). Cellular proliferation induced by arsenic may be due to dysregulation of positive and negative proliferation regulators.

Cells that become terminally differentiated can no longer divide. Murine preadipocytes are stimulated to differentiate after exposure to insulin and dexamethasone and these cells begin to accumulate lipid in vesicles that can be observed microscopically. The insulin/dexamethasone-induced differentiation of murine preadipocytes is inhibited by arsenite at noncytotoxic doses (Trouba et al., 2000b). This occurs either after an 8-week exposure to arsenite, or when arsenite is added to the insulin/dexamethasone stimulus. Removal of arsenite after the 8-week exposure renders the cells hyperresponsive to mitogenic stimulus. Also, the lipid vesicles in terminally differentiated adipocytes show signs of fragmentation after exposure to arsenite. It appears that arsenite is able dysregulate the equilibrium between proliferation and differentiation in the murine adipocytes.

In order for cells to proliferate, the cell cycle must not be arrested. Cyclin D is a protein required for cell cycle progression through G1, and in concert with other proteins (cdk4 and 6), inactivates pRb, the major protein responsible for antigrowth control. Cyclin D expression is increased in normal human fibroblasts after exposure to a low dose (0.1 μM) of arsenite for 14 days (Vogt and Rossman, 2001). Arsenite appears to have an effect on the cell cycle, which may alter cell proliferation.

7.6. Co-carcinogenesis

The interaction of As_2O_3 with benzo(a)pyrene (B(a)P), a carcinogenic polycyclic aromatic hydrocarbon found in tobacco smoke, was examined by Pershagen et al. (1984). The two chemicals were administered intratracheally at dose levels of 3 mg As/kg for As_2O_3 and 6 mg/kg for B(a)P. As observed in the tumorigenicity studies of inorganic arsenic after intratracheal administration, survival during the 15-week dosing regimen of this study raises some concern over the results. The

percentage of animals that survived the 15-week dosing regimen were: control, 78%; As_2O_3, 73%; B(a)P, 84%; $As_2O_3 + B(a)P$, 62%. In all dose groups (As_2O_3, B(a)P, $As_2O_3 + B(a)P$), papillomas, adenomas and carcinomas were observed in the respiratory tract (larynx, trachea, bronchi and lungs). When animals were exposed to both chemicals, there appeared to be a positive interaction in the development of respiratory tract carcinomas. Carcinomas were found in 25 out of 54 animals exposed to both chemicals, 14 out of 40 animals administered B(a)P, and 3 out of 47 animals administered As_2O_3. The $As_2O_3 + B(a)P$ group also had more pulmonary lesions (adenomas, papillomas, adenomatoid lesions) than in animals treated with each chemical alone.

Arsenite is cocarcinogenic with UV radiation in mouse skin (Rossman et al., 2001). Mice exposed to arsenite (10 mg/l) in their drinking water and UV radiation had a 2.4-fold increase in tumor yield compared to mice exposed to UV radiation alone. Tumors arose earlier in the co-exposed group, and the tumors were also larger and more invasive in this group than in the group exposed only to UV radiation.

7.7. Tumor promotion

Arsenite administered in drinking water does not promote tumors in the skin of wild-type mice topically treated with 3-methylcholanthrene (Milner, 1969) or in TG.AC mice, a transgenic mouse strain with genetically initiated skin (Germolec et al., 1997).

However, DMA^V, a metabolite of inorganic arsenic, is a tumor promoter (Yamamoto et al. 1995; Yamanaka et al., 1996; Wanibuchi et al., 1996). This has been observed in both rats and mice in multiple organs (rat-urinary bladder, kidney, liver, and thyroid gland; mice-lung). Lung tumors in the rat, however, are not promoted by DMA^V (Seike et al., 2002). The basic protocol in these studies is that the rodents are administered an initiator(s), followed by exposure to DMA^V in drinking water (2–400 ppm). Tumors begin to be promoted at levels of 10 ppm. However, it is questionable whether the levels of DMA used in the promotion studies would be attained in vivo after exposure to inorganic arsenic at doses that are not acutely toxic.

8. Summary

Arsenic is an environmental chemical of toxicological concern today. The metabolism of this metalloid has an important role in its toxicological effect. The recent evidence showing the higher acute toxicity of MMA^{III} than arsenite in vitro and in vivo, the carcinogenic and tumor promoting effects of DMA^V, and the direct genotoxic action of MMA^{III} and DMA^{III} in vitro suggests that the methylation of arsenic may not solely be a detoxication mechanism. These trivalent methylated arsenicals are also detected in the urine of individuals exposed to inorganic arsenic. Their MNA^{III} and DNA^{III} tissue levels are unknown, but may be high enough to cause an adverse effect. Information on the tissue dosimetry of the organic arsenicals (particularly in the trivalent form if possible) after exposure to inorganic arsenic, as well as in vivo toxicology studies with the trivalent organic arsenicals, are of critical importance. Improved analytical techniques will be required to examine the valency of the methylated arsenicals in vivo.

The underlying mechanism of toxicity for trivalent arsenic, the interaction with critical vicinal sulfhydryl groups, appears to be well-founded on a biochemical basis. On the other hand, the mechanism for pentavalent arsenic, the replacement of phosphate, is not firmly set, and additional research in this area is needed. However, the major portion of the toxic effects of arsenate may actually be due to its reduction to arsenite.

While epidemiology studies indicate that arsenic is a human carcinogen, the mechanism of action is not known. Because arsenic can elicit many diverse effects, more than one mechanism may be involved in its carcinogenic effect. The lack of a viable animal model as well as the inability for arsenic to be a "classical" mutagen (point mutations) has hindered research on defining a mechanism of action for arsenic. Certainly the results of Ng et al. (1999) warrant confirmation. The use of transgenic models (e.g. TG.AC,

K6/ODC) and in vivo studies using inorganic or organic arsenic as a tumor promoter/co-carcinogen offers promising results. Also, the evidence that the inorganic arsenic metabolites MMA^{III} and DMA^{III} are genotoxic by directly damaging DNA, and additional research in this area, will benefit our understanding of a mechanism of action for arsenic.

Arsenic in many ways causes the cell to signal or alters normal signaling. Oxidative stress, which can damage DNA, induces cells to signal a response by turning on heat-shock protein production. Cellular DNA is both hypo- and hypermethylated in the presence of arsenic, which affects whether or not the DNA is transcribed. DNA that is transcribed (or not) may result in a cascade of events, some of which may be adverse. Growth factors are secreted by cells in the presence of arsenic, and cells are stimulated to proliferate, all of which requires an initial signal, and arsenic appears to be a good candidate for this effector. DNA repair activity is inhibited by arsenic, but the inhibition is not a direct action of arsenic on the repair enzymes. It appears that some type of effect on the signal to repair the DNA is produced by arsenic. Certainly a good portion of the most recent research has been on the effect of arsenic on cell signaling. The development of molecular biological tools, such as gene arrays, will most assuredly assist in understanding a mechanism of action of arsenic.

The major goal of research described here is to understand how arsenic causes an adverse effect. While many, if not all of these adverse effects induced by arsenic are known, there is still a question about the risk to individuals who are exposed to arsenic, as well as the dose needed for these effects to develop. A definitive understanding of the mechanism of action of arsenic will allay any uncertainties associated with the risk assessment for this chemical.

9. Disclaimer

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