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REVIEW

Recent Advances in Arsenic Carcinogenesis: Modes of Action, Animal Model Systems, and Methylated Arsenic Metabolites[1]

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Recent advances in our knowledge of arsenic carcinogenesis include the development of rat or mouse models for all human organs in which inorganic arsenic is known to cause cancer–skin, lung, urinary bladder, liver, and kidney. Tumors can be produced from either promotion of carcinogenesis protocols (mouse skin and lungs, rat bladder, kidney, liver, and thyroid) or from complete carcinogenesis protocols (rat bladder and mouse lung). Experiments with p53$^{+/-}$ and K6/ODC transgenic mice administered dimethylarsinic acid or arsenite have shown some degree of carcinogenic, cocarcinogenic, or promotional activity in skin or bladder. At present, with the possible exception of skin, the arsenic carcinogenesis models in wild-type animals are more highly developed than in transgenic mice. Recent advances in arsenic metabolism have suggested that methylation of inorganic arsenic may be a toxification, rather than a detoxification, pathway and that trivalent methylated arsenic metabolites, particularly monomethylarsonous acid and dimethylarsinous acid, have a great deal of biological activity. Accumulating evidence indicates that these trivalent, methylated, and relatively less ionizable arsenic metabolites may be unusually capable of interacting with cellular targets such as proteins and even DNA. In risk assessment of environmental arsenic, it is important to know and to utilize both the mode of carcinogenic action and the shape of the dose–response curve at low environmental arsenic concentrations. Although much progress has been recently made in the area of arsenic's possible mode(s) of carcinogenic action, a scientific concensus has not yet been reached. In this review, nine different possible modes of action of arsenic carcinogenesis are presented and discussed–induced chromosomal abnormalities, oxidative stress, altered DNA repair, altered DNA methylation patterns, altered growth factors, enhanced cell proliferation, promotion/progression, gene amplification, and suppression of p53.

Key Words: **arsenic; monomethylarsonic acid; dimethylarsinic acid; carcinogenesis; K6/ODC transgenic mice; p53$^{+/-}$ mice.**

Substantial scientific progress has been made recently in the area of arsenic metabolism, pharmacokinetics, modes of carcinogenic action, and the development of animal models of arsenic carcinogenesis in both normal rodents and transgenic mice. This review of arsenic carcinogenesis will discuss the modes of action, existing animal models, and the importance of methylated arsenic metabolites. Additional, more speculative modes of arsenic carcinogenesis could have been included in this article. Some of the included modes of action appear to be predominantly genotoxic (e.g., chromosomal abnormalities, oxidative stress, and gene amplification) while others seem to be more nongenotoxic (e.g., altered growth factors, enhanced cell proliferation and promotion of carcinogenesis, and altered DNA repair). Whatever the better label for a mode of carcinogenic action is, the dose–response relationship at low arsenic concentrations for any of these nine putative modes of arsenic carcinogenesis is not known.

The present deficiency of scientific knowledge in the areas of modes of carcinogenic action for arsenic and the true shape of the dose–response curve at low arsenic concentrations (e.g., corresponding to 2, 5, 10, 20, and 50 ppb in the drinking water supply of human beings) negatively effects the process of regulating environmental arsenic exposures. Without adequate scientific knowledge on arsenic carcinogenesis, default assumptions will be used for both the mode of carcinogenic action and the model used for extrapolation from the experimentally observable ranges to human environmental exposures. Scientific knowledge may then take a back seat to economic pressures, political ideology, and litigative outcomes.

PUTATIVE CARCINOGENIC MODES OF ACTION FOR ARSENIC

Chromosome Abnormalities

As arsenicals are usually found to be effective for clastogenicity and low in point mutagenicity, this is a favorite putative

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carcinogenic mode proposed by many individuals. There are many positive reports of arsenic-induced chromosomal aberrations, including micronuclei and sister chromatid exchanges, found in studies in humans and rodents both *in vivo* and *in vitro*.

Dimethylarsinic acid (DMA) causes several genotoxic or clastogenic effects, including single strand breaks, formation of apurinic/apyrimidinic sites, DNA base damage and oxidative base damage, DNA–protein crosslinks, chromosomal aberrations, and aneuploidy. Higher concentrations of DMA are needed to demonstrate clastogenicity in comparison to lower concentrations of arsenite. Li and Rossman (1989) have suggested that the clastogenicity of arsenite is due to the high affinity of arsenic for sulfhydryl groups. Interactions of arsenic with the protein tubulin and spindle formation and function are areas that have attracted interest.

In humans' drinking water containing 400 μg/L of arsenic, an increased frequency of chromosome aberrations was found in peripheral lymphocytes (Beckman *et al.*, 1977; Nordenson *et al.*, 1978 and Petres *et al.*, 1977). An increased frequency of micronucleated urothelial exfoliated cells was also found in humans' drinking water with high levels of arsenic (Warner *et al.*, 1994). A third study of exposed humans found increased numbers of micronuclei in exfoliated epithelial cells from both the bladder and oral cavity (Gonsebatt *et al.*, 1997).

The connection between the demonstrated clastogenicity of arsenic and how exactly this may cause carcinogenicity in five different human organs is not well understood. Rather than continuing just to demonstrate clastogenicity in different cellular or animal systems, more effort should be spent trying to illuminate the subsequent mechanistic steps of arsenic carcinogenesis that may occur after clastogenicity.

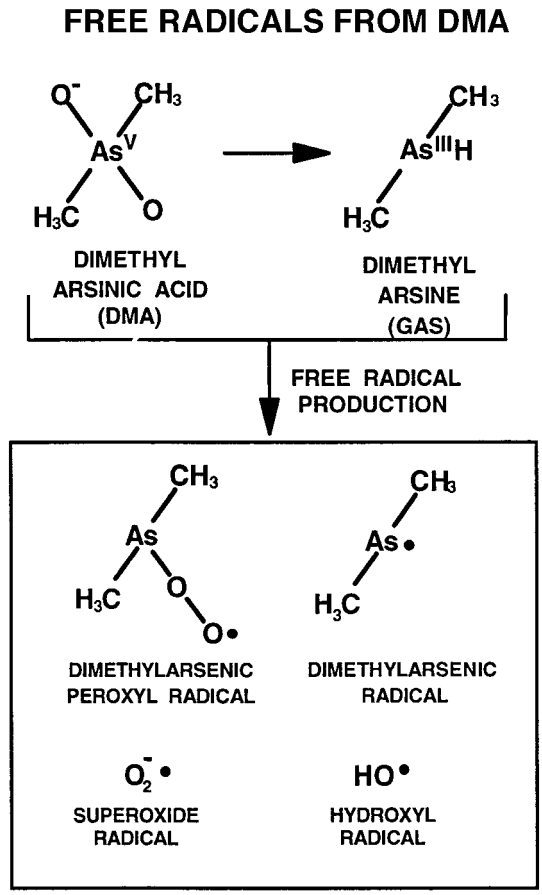

FIG. 1. Free radicals from DMA. Pentavalent DMA may be reduced to a trivalent arsenic form, dimethyarsine. This minor pathway of arsenic metabolism may generate free radicals such as dimethylarsenic radical, dimethylarsenic peroxy radical, or indirectly generate superoxide and hydroxy radical. Modified from Yamanaka *et al.* (1990).

Oxidative Stress

Oxidative stress is a relatively new theory of arsenic carcinogenesis. Since about 1990, additional data supporting this theory and greater scientific acceptance of this mode of action have continued to accrue. The first oxidative stress theory of arsenic carcinogenesis that included a detailed arsenic metabolic pathway was presented by Yamanaka *et al.* (1990). Dimethylarsine (a trivalent arsenic form) is a minor *in vivo* metabolite of DMA (a pentavalent arsenic form) produced by a process of reduction *in vivo* (Yamanaka and Okada, 1994). As summarized in Fig. 1, dimethylarsine can react with molecular oxygen forming a $(CH_3)_2As^{\bullet}$ radical and superoxide anion. This $(CH_3)_2As^{\bullet}$ radical can add another molecule of molecular oxygen and form the $(CH_3)_2AsOO^{\bullet}$ radical. Hydroxyl radical may be produced via cellular iron and other transition metals. From exposure to these free radicals, DNA damage such as DNA single-strand breaks can occur.

One striking advantage to the oxidative stress theory of arsenic carcinogenicity is that arsenic's ability to cause human cancer at high rates in the lungs, bladder, and skin can be partially explained. High partial pressures of oxygen are found in the lung. Human lungs may be an organ responsive to arsenic carcinogenesis because of the high partial pressure of oxygen and the fact that dimethylarsine, a gas, is excreted via the lungs (Yamanaka and Okada, 1994). Human bladder may be an organ responsive to arsenic carcinogenesis because of the high concentration of DMA and MMA that is stored in the lumen of the bladder and the amount of DMA(III), dimethyl-

[2] Abbreviations used: DMA (the pentavalent form), dimethylarsinic acid; DMA(III), dimethylarsinous acid; DMBA, 7,12-dimethylbenz[a]anthracene; GM-CSF, granulocyte macrophage-colony stimulating factor; K6/ODC, transgenic mice which overexpress a truncated ODC protein in hair follicle keratinocytes; MMA (the pentavalent form), monomethylarsonic acid; MMA(III), monomethylarsonous acid; ODC, ornithine decarboxylase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PARP, human poly(ADP-ribose)polymerase; ROS, reactive oxygen species; SAM, *S*-adenosylmethione; Tg.AC, female homozygous transgenic mice with a fetal zeta-globulin promoter fused to the v-Ha-*ras* structural gene (with mutations at codons 12 and 59) and linked to a simian virus 40 polyadenylylation/splice sequence; TGF-α, tissue growth factor-α; TMA(III), trimethylarsine; TMAO, trimethylarsine oxide (the pentavalent form); TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

arsine, or MMA(III) that might be generated by reductive processes. Skin localizes and stores arsenic because of its high keratin content, thus there is a pharmacokinetic reason why skin may be so responsive to arsenic exposure. Skin seems unusually sensitive to both arsenic's toxic and carcinogenic effects. Two human organs that respond at much lower rates to arsenic carcinogenesis are the liver and the kidney. DMA is produced via oxidative methylation in the liver and the kidney is exposed to high concentrations of DMA as it filters DMA into the urine.

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical can directly or indirectly damage cellular DNA and protein. Among these ROS, hydroxyl radical is generally assumed to be the critical reactive species that directly attacks DNA. For hydroxyl radical to be involved in arsenic carcinogenesis, a free transition metal (such as iron) is normally thought to be required for Haber Weiss type processes to cause DNA damage. When tested as releasers of iron from ferritin, (a) methylated arsenic forms were more active than arsenate or arsenite, (b) the trivalent arsenic forms were more active than the corresponding pentavalent arsenic forms, and (c) DMA(III) was by far the most active releaser of iron from ferritin (Ahmad *et al.*, 2000). A combined *in vitro* exposure to both ascorbic acid (a well known iron releaser) and DMA(III) resulted in a large synergistic (greater than additive) increase in iron released from ferritin (under either aerobic or anaerobic conditions) and also in a large synergistic increase in DNA damage (Ahmad *et al.*, 2000). Arsenite administration induces hepatic and renal heme oxygenase isoform 1 in rats (Kitchin *et al.*, 1999). Heme oxygenase induction results in the production of carbon monoxide, biliverdin, and free iron.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is one of the major ROS-induced DNA damage products and is used as a biomarker of oxidative stress to DNA. In mice gavaged with 720 mg/kg of DMA, urinary 8-OHdG levels (obtained by sampling from the urinary bladder) were increased to about 3, 3, and 8 times control levels at 3, 6, and 9 h after treatment, respectively (Yamanaka *et al.*, 2001). In a long-term rat carcinogenensis study, hepatic 8-OHdG levels were increased in DMA-treated rats, suggesting an elevated rate of free radical attack on DNA (Wanibuchi *et al.*, 1997).

In a human study, 28 cases of arsenic-related skin neoplasms and keratosis as well as 11 cases of arsenic-unrelated Bowen's disease were studied. By immunohistochemistry, 22 of 28 (78%) arsenic-related human skin samples were 8-OHdG positive (Matsui *et al.*, 1999). In the arsenic-unexposed group only 1 of 11 Bowen's disease samples (9%) gave a positive immunohistochemistry reaction with the 8-OHdG antibody procedure. In addition, in the arsenic-exposed group, 4 of 5 deparaffined human skin tumor samples tested showed detectable arsenic by neutron activation analysis. Thus, both the causative chemical (arsenic) and a putative intermediate (8-OHdG) in an oxidative stress mode of carcinogenesis were found together in the same human skin tumor samples (Matsui *et al.*, 1999).

Positive results were obtained in an electron spin resonance experiment utilizing mice (Liu *et al.*, 2000). Mice were given either 100 μmol/kg of arsenite or 500 μmol/kg of arsenate by the sc route. The animals were also given a spin trap agent at the same time. Thirty minutes after the arsenic exposure, the mouse livers were removed and extracted to separate the spin trap agent from other biological material. Both arsenite and arsenate gave positive results, with the stronger electron spin resonance signals coming from arsenite-exposed mice. These data directly demonstrate that some free radicals are produced in mice after acute exposure to inorganic arsenic (Liu *et al.*, 2000).

In cultured human lymphocytes exposed to arsenite, increased sister chromatid exchange frequency was antagonized by the addition of superoxide dismutase and catalase (Nordenson and Beckman, 1991). Induction of micronuclei in CHO-K1 cells by 20 μM arsenite was antagonized by either nitric oxide synthase inhibitors, superoxide dismutase, or uric acid (Gurr *et al.*, 1998). These results suggest that some clastogenic effects of arsenic are mediated via free radicals (e.g., peroxynitrite, superoxide, hydrogen peroxide, and possibly free iron).

Altered Growth Factors

In primary human keratinocytes, arsenite exposure increased the mRNA transcripts and secretion of transforming growth factor-α (TGF-α), granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) (Germolec *et al.*, 1997). Increased keratinocyte proliferation was also observed.

In a subsequent study with human keratinocytes, exposure to three trivalent arsenicals, arsenite, MMA(III), and the DMA(III)-glutathione conjugate, resulted in increased secretion of GM-CSF, TNF-α, and interleukin-6 as well as increased cell proliferation (Vega *et al.*, 2001). The authors suggest that arsenic exposure could increase the production of ROS, activation of transcription factors (e.g., AP-1, c-*fos*, and NF-κB), and oversecretion of proinflammatory and growth promoting cytokines, resulting in increased cell proliferation and finally carcinogenesis. At present, data suggesting altered growth factors as a mode of arsenic carcinogenesis are much more highly developed in skin models than for other tissues such as lung or bladder. The possible role of growth factors, cell proliferation, and other epigenetic modes of action in arsenic carcinogenesis is discussed by Simeonova and Luster (2000).

Cell Proliferation

Increased ornithine decarboxylase (ODC) activity is often interpreted as a biomarker for cell proliferation. Arsenite exposure in rat liver (1.6 mg/kg) (Brown and Kitchin, 1996) and DMA exposure in rat liver (at 10 ppm) (Wanibuchi *et al.*, 1997) and in rat kidney (at 100 ppm DMA) (Yamamoto *et al.*,

TABLE 1
DMA Carcinogenesis in Rodents

	Species	Complete carcinogen	Promoter	Lowest effective DMA concentration in water (ppm)	Reference
Skin	Mouse	—	Yes	1000	Yamanaka *et al.*, 2000
Lung	Mouse	—	Yes	200	Yamanaka *et al.*, 1996
	Mouse	Yes	—	400	Hayashi *et al.*, 1998
Bladder	Rat	—	Yes	50	Yamamoto *et al.*, 1995
	Rat	—	Yes	25	Wanibuchi *et al.*, 1996
	Rat	Yes	—	50	Wei *et al.*, 1999
	Rat	Yes	—	100 (in diet)	Life Science Research, 1989
Kidney	Rat	—	Yes	200	Yamamoto *et al.*, 1995
Liver	Rat	—	Yes	200	Yamamoto *et al.*, 1995
	Rat	—	Yes	50	Wanibuchi *et al.*, 1997
Thyroid	Rat	—	Yes	400	Yamamoto *et al.*, 1995

1995) have resulted in increased ODC activity. Increased cell proliferation following arsenic exposure has been demonstrated in skin (Germolec *et al.*, 1997 (at 0.001 μM), keratoses in many human studies), and urinary bladder (Popovicova *et al.*, 2000 (at 500 ppm); Arnold *et al.*, 1999 (at 40 ppm)).

Two common causes of cell proliferation are mitogenic stimulation and cell toxicity and death followed by compensatory regeneration. Errors of replication resulting from unrepaired DNA damage present at the time of DNA replication can result in mutation of the genetic material. In cases in which cell proliferation is the upstream cause of the eventual mutation in DNA, it is difficult to decide if the best mechanistic label to apply to such a sequence of events is nongenotoxic or genotoxic.

Promotion and/or Progression in Carcinogenesis

In carcinogenesis, the terms promoter and progressor are operationally defined. These two terms are more difficult to translate into particular mechanistic steps than is carcinogenic initiation. Carcinogenesis can be thought of in terms of a mutational/cellular proliferation cascade model (Moolgavkar 1986; Clayson and Kitchin, 1999). In a more holistic approach to understanding carcinogenesis, Hanahan and Weinberg (2000) state that the hallmarks of cancer include self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Current theories of arsenic carcinogenesis have little or nothing to say about the latter three of these important factors in carcinogenesis. As shown in Table 1, there is positive evidence for DMA promotion of carcinogenesis in skin, lung, bladder, kidney, liver, and thyroid. Much of this promotional activity may be due to increased cell proliferation rates.

Cell proliferation is an important driving force in promotion of carcinogenesis; mutation is more important in initiation and in progression in which increasing malignancy and metastatic potential develops in one cell of the large number of existing cells of intermediate malignancy.

Altered DNA Repair

Arsenite is known to inhibit more than 200 enzymes (Abernathy *et al.*, 1999). That a sulfhydryl active agent like arsenite would inhibit this many enzymes is not surprising. Of the possible tens of thousands of cellular enzymes, almost all have covalent disulfide linkages (cystine), while a lower number of proteins have one or more free cysteine moieties along the polypeptide chain. After translation, proteins undergo complex metabolism, redox changes, adduction with other molecules (e.g., sugars), and binding with other cellular entities. A small trivalent oxyanion like arsenite with an unshared pair of 4 s electrons has many opportunities for protein binding, conformational alteration of protein structure, and enzymatic inhibition. Arsenicals with this capability include arsenite, MMA(III), DMA(III), TMA(III), and dimethylarsine.

Early work on DNA repair enzymes showed that DNA ligases I and II were both inhibited by arsenite (Li and Rossman, 1989; Lee-Chen *et al.*, 1993). Later work with purified human DNA repair enzymes showed that arsenite actually increased the activities of DNA polymerase beta, O^6-methylguanine-DNA methyltransferase and DNA ligases I, II, and III (Hu *et al.*, 1998). Human poly(ADP-ribose)polymerase (PARP) activity is also inhibited by arsenite (Yager and Wiencke, 1997).

Although trivalent arsenicals have a reasonable affinity for single sulfhydryl groups, trivalent arsenic can bind with a higher binding affinity to two proximate sulfhydryl groups. These nearby dithiols are relatively rare among proteins in general but are fairly common among certain DNA-binding proteins, transcription factors, and DNA-repair proteins. When certain amino acid sequences are complexed to endogenous zinc, these proteins are called "zinc finger" proteins. Some examples of zinc finger DNA-repair proteins are UVRA (Hu-

sain *et al.*, 1986), PARP (Cherney *et al.*, 1987), RAD-18 (Jones *et al.*, 1988), and XPAC (Tanaka *et al.* 1990).

The theory that altered DNA repair is the cause of arsenic carcinogenesis is particularly attractive because trivalent arsenic species, such as arsenite, can bind strongly to dithiols as well as free sulfhydryl groups. Such protein binding could induce inhibited DNA repair, mutation in key genetic sites, or increased cell proliferation which can then lead to subsequent mutation via inhibited DNA repair.

p53 Gene Suppression

The immortalized keratinocyte cell line HaCaT, which contains two UV-induced mutations in the p53 gene, was exposed to arsenite (1, 10, or 100 nM) (Hamadeh *et al.*, 1999). After 2 to 4 days of arsenite exposure, p53 protein levels were decreased. Concomitant arsenite-induced increase in mdm2 levels seemed to cause the observed p53 protein level decrease. As p53 is the guardian of the genome, lessened p53 protein content and function could cause mutations to accumulate at a faster rate in organisms exposed to arsenite, leading eventually to carcinogenesis (Hamadeh *et al.*, 1999). In contrast, *in vitro* arsenite exposure (1–50 uM) increased the p53 protein levels found in three (HeLa, Jurkat, and a lymphoblast cell line transformed with Epstein–Barr virus) of four cell lines investigated (C-33A cells did not respond) (Salazar *et al.*, 1997).

In arsenic-related skin cancers from Taiwan, p53 mutations were found in 39% of cases with Bowen's disease, 29% of the cases with basal cell carcinoma, and 56% of the cases with squamous cell carcinomas (39 cases total) (Hsu *et al.*, 1999). In arsenic-related skin lesions arising from therapeutic use of arsenic, no indications of p53 mutations were found among the 18 basal cell carcinomas or 2 squamous cell carcinomas in 8 patients studied (Castren *et al.*, 1998). However, in premalignant lesions, mutations were found in 30% of the skin lesions following arsenic exposure. Accumulation of p53 protein was found in 78% of the lesions from cases with arsenic exposure (Castren *et al.*, 1998). In contrast, in Australian patients exposed to therapeutic use of arsenic, arsenic-related basal cell carcinomas expressed p53 less often and with lower staining intensities than did control patients exhibiting sporadic basal cell carcinomas (Boonchai *et al.*, 2000).

Altered DNA Methylation Patterns

Most mammals biomethylate arsenic. In most species, inorganic arsenic consumes the methyl groups of two molecules of *S*-adenosylmethionine (SAM) in its metabolism to DMA (Fig. 2). Some species, such as the guinea pig, the chimpanzee, and the marmoset have extremely low rates of arsenic methylation, to the point where these species have been described as "nonmethylating." Rats produce some urinary trimethylated arsenic species (trimethylarsine oxide (TMAO); S. Fukushima, personal communication) from administered DMA.

DNA is also methylated and the importance of this methylation has long been appreciated. In human lung adenocarcinoma A549 cells exposed to arsenite (0.08 to 2.0 uM) or arsenate (30 to 300 uM), a hypermethylation of a promoter region of the p53 gene was observed (Mass and Wang, 1997). Other researchers (Zhao *et al.*, 1997) have found hypomethylation, not hypermethylation, of DNA in a rat epithelial cell line (TRL 1215) exposed to arsenic. They suggest DNA hypomethylation could be capable of committing cells toward a carcinogenic pathway. DNA methylation state changes could lead to altered gene expression and this could lead to carcinogenesis. What is more difficult to prove and is not presently known is how DNA hypo- or hypermethylation would act through the intervening steps of arsenic carcinogenesis to finally result in an invasive tumor.

The methylation state of DNA following arsenite exposure was further investigated in human lung A549 cells and in three human kidney cell lines (UOK123, UOK109, and UOK121) (Zhong *et al.*, 2001). A total of eight differentially methylated DNA fragments were isolated using methylation-sensitive arbitrarily primed PCR. DNA sequence studies found that two DNA fragments were repeat sequences of mammalian apparent LTR retrotransposons, five were putatively identified as promoter-like sequences, and one DNA fragment was not identifiable. Six of the DNA fragments were hypermethylated; two were hypomethylated. In A549 cells, elevations were found in DNA methyltransferase mRNA and its enzymatic activity (up to three times control levels after 2 μM arsenite exposure for 2 weeks).

Gene Amplification

Lee *et al.* (1988) described arsenic-induced gene amplification of the dihydrofolate reductase gene in mouse 3T6 cells. Cells had a 2- to 11-fold increased copy number of the dihydrofolate reductase gene. In causing gene amplification, arsenite was active in the 0.2 to 0.8 μM range and arsenate was active between 1 and 4 μM. Higher concentrations of arsenite or arsenate decreased cellular survival markedly but also had much higher relative rates of gene amplification. Other studies have also found arsenic-induced dihydrofolate reductase gene amplification in SHE cells (Woloson, 1990) and in arsenite-resistant trypanosomes (Katakura and Chang, 1989).

Summary

The modes of carcinogenic action of arsenic will remain an area of active scientific research and disagreement. At this time, three modes of action for arsenic carcinogenesis have a degree of positive evidence, both in experimental systems (animal and human cells) and in human tissues that warrant preeminence–chromosomal abnormalities, oxidative stress, and a continuum of altered growth factors → cell proliferation → promotion of carcinogenesis. The remaining possible modes of carcinogenic action for arsenic (progression of carcinogenesis, altered DNA repair, p53 suppression, altered

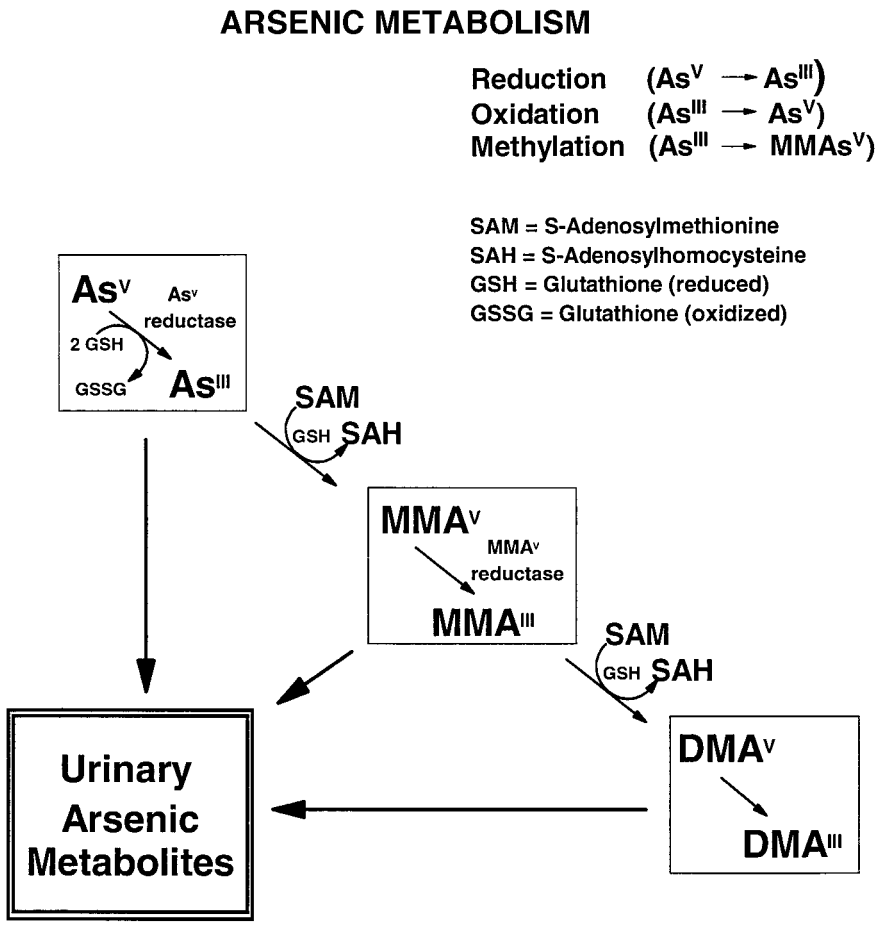

FIG. 2. A simplified scheme of overall arsenic metabolism in many mammals including humans. Reduction from pentavalent to trivalent arsenic states may occur nonenzymatically via glutathione or enzymatically. Oxidation and methylation are coupled in arsenic metabolism with the trivalent arsenic form as the substrate and a more methylated pentavalent arsenic form as the product. As(V), As(III) MMA, MMA(III), DMA (the major form in many mammals), and DMA(III) are found in human urine. Humans excrete a relatively high amount of MMA in their urine. In rats, some arsenic is further metabolized to a form with three methyl groups, TMAO. Only recently has it been possible to separate the two different valence states of MMA and DMA, hence, they are grouped together within a single box in this figure. Some forms of arsenic can reversibly change valence state from pentavalent to trivalent and back again (e.g., arsenate ↔ arsenite).

DNA methylation patterns, and gene amplification) do not have as much evidence for them, particularly animal evidence *in vivo*, positive evidence in human cells, or human data from case or population studies.

Chromosomal abnormalities can be easily caused because of the tendency for trivalent arsenic forms to interact and disrupt the normal functioning of tubulin and spindles. It is the second, third and fourth steps of cancer causality that are weakest in the chromosomal abnormalities theory of arsenic carcinogenesis.

Oxidative stress is a mode of carcinogenic action for arsenic that works particularly well in the lungs and bladder, but not as well in skin. The oxidative stress theory partially depends on the ability of DMA or MMA metabolites to form free radicals. Alternatively, the inorganic forms of arsenic could directly generate free radicals. This can occur because arsenic changes oxidation states from trivalent to pentavalent depending on the exterior chemical environment. Recent mouse experiments showed rapid formation of free radicals after administration of arsenate or arsenite (Liu *et al.*, 2000). Formation of 8-OHdG in mouse bladder (Yamanaka *et al.*, 2001) and human skin (Matsui *et al.*, 1999) suggests involvement of oxidative stress in arsenic carcinogenesis.

The combined theory of altered growth factors → cell proliferation → promotion of carcinogenesis is an excellent choice for a carcinogenic mode of action for arsenic. The increases in concentrations of growth factors is a mitogenic pathway to cell proliferation and eventually promotion of carcinogenesis. Cell death, coming from a trivalent form of arsenic, can also lead to compensatory cell regeneration and eventually to carcinogenesis. All three components of this combined mode of action have been demonstrated in one or more systems: altered growth factors (in human keratinocytes); mitogenesis (in human keratinocytes); cell death (in human hepatocytes *in vitro*, Petrick *et al.*, 2000; in rat bladder epithelium *in vivo*, Cohen *et*

al., 2001; cell proliferation (in human keratinocytes and rodent bladder cells and intact human skin), and promotion of carcinogenesis (in mouse skin and lung and in rat bladder, kidney, liver, and thyroid, see Table 1).

ANIMAL MODELS OF ARSENIC CARCINOGENESIS

Studies Showing No Tumors

Arsenite and arsenate are generally considered to have tested negative in standard carcinogenicity bioassays (NRC, 1999). Negative results have been obtained in mice (at dietary concentrations of 250 ppm for arsenite and 400 ppm for arsenate), rats (416 ppm arsenite in the diet), beagles (arsenite or arsenate at up to 125 ppm in the diet), and cynomologus monkeys (arsenate at 0.1 mg/kg per day for 5 days per week for 15 years) (NRC, 1999). In an unpublished study, hamsters were exposed for 425 days to biweekly administration of As_2O_3 (0.3 mg/kg, (this is not likely to be close to the maximum tolerated dose)) and/or TiO_2 (15 mg/kg) via intratracheal exposure (volume 0.1 ml) but no lung masses were seen on gross examination (L. L. Hall, personal communication).

Animal Models of Arsenic (DMA) Carcinogenesis

Urinary bladder. Four positive studies of DMA as either a promoter or a complete carcinogen in rat bladder are summarized in Table 1. An indication that rat bladder was responsive to DMA-induced carcinogenesis came from the Yamamoto *et al.* (1995) report. They utilized multiple initiators of carcinogenesis at the early stage of chemical exposure to start the carcinogenic processes in several organs. Subsequently, the F344/DuCrj rats were exposed to either 50, 100, 200, or 400 ppm of DMA in their drinking water. Bladder cancer resulted in all four of the DMA-exposed groups. Rat bladder tumors were also obtained when only one initiator, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine was followed by 25 ppm DMA exposure (Wanibuchi *et al.*, 1997). Wei *et al.* (1999) tested DMA as a complete carcinogen and found it to be carcinogenic to rat urinary bladder at 50 and 200 ppm.

In a structure–activity study, rats initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine developed 0.4, 0.3, 1.3, 3.8, and 0.9 bladder tumors per rat after being given drinking water containing control, arsenite (17.3 ppm), MMA (187 ppm), DMA (184 ppm), or TMAO (182 ppm), respectively (Wanibuchi *et al.*, 2000). The rat bladder tumor incidence was elevated at the $p < 0.05$ level for all three methylated arsenicals, with DMA clearly the most active chemical.

In another study, female F344 rats exposed for 2 years to up to 100 ppm DMA in their food developed dose-related transitional cell bladder tumors with bladder hyperplasia observed in both sexes. Life Sciences Research of Israel performed this study in support of the U.S. EPA pesticide registration for cacodylic acid (EPA, 1994), however, the results of this study were not initially widely known outside of regulatory circles. Control female rats had 0/59 incidence of bladder papillomas and carcinomas, while female rats exposed to 100 ppm DMA had 10/58 incidence (17%, with a $p < 0.01$). Of the female rats that responded to DMA exposure with bladder tumors, six were classified as carcinomas and four were classified as papillomas. Female B6C3F1 mice exposed to up to 500 ppm DMA in their drinking water showed only an increase in fibrosarcomas in multiple organs.

Scanning electron microscopy of bladder epithelium of female F344 rats administered 100 ppm DMA in the diet showed ropy microridges, extensive pitting, increased separation of epithelial cells, exfoliation, and necrosis (Cohen *et al.*, 2001). These changes are consistent with cell death and regenerative hyperplasia as the cause of DMA-induced bladder carcinogenesis.

Based on these multiple studies showing DMA to be both a promoter of carcinogenesis (Yamamoto *et al.*, 1995; Wanibuchi *et al.*, 1997) and a complete carcinogen (Life Science Research, 1989; Wei *et al.*, 1999), rat bladder is the most studied and probably best understood experimental model of arsenical carcinogenesis (Table 1).

Lung. Human lung cancer can result from arsenic exposure either via drinking water or inhalation. Male ddY mice initiated with 4-nitroquinoline 1-oxide and subsequently exposed to 200 or 400 ppm DMA for 25 weeks in their drinking water developed lung tumors that were described as adenocarcinomas and adenosquamous carcinomas (Yamanaka *et al.*, 1996).

A/J mice were given 50, 200, or 400 ppm DMA in drinking water for either 25 or 50 weeks (Hayashi *et al.*, 1998). At 25 weeks there were no DMA-related pulmonary tumors. Alveolar adenomas were not increased by DMA administration at 50 weeks. In mice given 400 ppm DMA for 50 weeks, an elevated mean number of lung tumors per mouse was observed (1.36 versus 0.5 for controls, $p < 0.05$). Increased numbers of lung hyperplasia, papillary adenomas, and adenocarcinomas were observed after treatment with 50, 200, or 400 ppm of DMA for 50 weeks. In this experiment, DMA is acting as a complete carcinogen in A/J mice known to be susceptible to developing pulmonary tumors.

Skin. Despite the high responsiveness of human skin to both arsenic's toxicity and carcinogenicity, wild-type rodent skin models of carcinogenicity have not been very responsive to arsenic. Mouse skin is well known to be sensitive to many chemical carcinogens; rat skin is extremely unresponsive to chemical carcinogenesis.

Mice were treated for 25 weeks with UVB light at 2 kJ/m^2 twice per week with or without exposure to 400 or 1000 ppm of DMA in the drinking water. All three treatment groups developed skin cancer, but the combination of UVB light and 1000 ppm of DMA in the drinking water induced more tumors per mouse during weeks 13 to 19 than did UVB light exposure alone. No statistically significant elevations in the percentage of tumor-bearing mice or tumors per mice occurred with the

combination of UVB and 400 ppm DMA treatment versus UVB treatment alone (Yamanaka *et al.*, 2000).

Liver. Liver was one of the four rat organs (the others were bladder, kidney, and thyroid) that developed tumors after multiple organ initiation and exposure to DMA as a promoter (Yamamoto *et al.*, 1995). Rat liver carcinogenesis can be studied in a two-stage system in which diethylnitrosamine is the initiator and DMA is the promoter. Concentrations of 25, 50, or 100 ppm DMA are active as promoters in this experimental system based on the area stained for glutathione *S*-transferase placental form-positive foci (Wanibuchi *et al.*, 1997). Rat hepatic ODC activity was increased after administration of both 10 and 50 ppm DMA in the drinking water (Wanibuchi *et al.*, 1997).

In a structure–activity study, rats initiated with diethylnitrosamine developed about 1.5, 2.5, 2.4, and 2.5 glutathione *S*-transferase placental form-positive foci/cm^2 following exposure to promotional regimens of control, MMA, DMA, or TMAO, respectively (Wanibuchi and Fukushima, 2000). The number of liver foci per area was statistically elevated at the $p < 0.05$ for all three methylated arsenicals.

Kidney. At concentrations of 200 or 400 ppm DMA in drinking water, rats initiated with a multiple initiator procedure developed kidney cancer (Yamamoto *et al.*, 1995). These kidney tumors were classified as adenomas, adenocarcinomas, renal cell tumors, or nephroblastomas.

Thyroid. At a concentration of 400 ppm DMA in drinking water, rats initiated with a multiple initiator procedure developed thyroid cancer. Interestingly, Sprague–Dawley rats exposed for 2 years to dietary concentration of 200 ppm MMA, the precursor of DMA, showed increased thyroid tumors in males (EPA, 1981). Five rodent organs that are responsive to DMA-induced cancer match the five human organs that develop cancer after inorganic arsenic exposure. The thyroid gland (also responsive to DMA in rats) is the only site not known to develop cancer after inorganic arsenic exposure in humans.

Summary. The pharmacokinetics and dynamics of administered pentavalent DMA in rats suggests (a) substantial DMA binding to hemoglobin that sequesters the DMA and increases its biological lifetime but not necessarily its access to targets needed to be carcinogenic, (b) an appreciable fraction of the DMA may be in the pentavalent state, (c) rapid excretion of unbound DMA, and (d) the likely active carcinogenic form of the administered DMA could be DMA(V), DMA(III), TMAO, or TMA(III). One research group has found that rats metabolize DMA to TMAO to a larger extent (about 30% of the administered DMA material) (S. Fukushima, personal communication) than do mice, hamsters, and humans (about 3 to 6% of administered DMA) (Kenyon and Hughes, 2001). Following DMA administration to rats, there are two predominant trivalent arsenicals that might be causal in carcinogenesis–DMA(III) and TMA(III).

After decades of not having an animal model of arsenic carcinogenesis, the scientific community was somewhat surprised by the 1995 report of DMA being a promoter of carcinogenesis in four different rat organs (Yamamoto *et al.*, 1995). While the scientific reaction to this major contribution was positive in many ways, some of the less positive reactions may have been based on the following four factors. First, it was anticipated that arsenite, not DMA would be the cause of arsenic carcinogenesis. Second, the use of a multiple organ initiation protocol caused some consternation in interpreting this study because (a) a multiple rather than a single initiator was used, (b) these initiators are complete carcinogens in some exposure protocols, and (c) the cumulative and interactive effects of using multiple initiators are not well known in the mutational sense. Third, the minimal drinking water concentrations of DMA needed to promote carcinogenesis (25 ppm) or to be a complete carcinogen (50 ppm) were higher than expected. Fourth, humans exposed to inorganic arsenic are limited by the toxicity and lethality of arsenite in how high DMA concentrations their tissue can reach.

Animals models of DMA-induced promotion of carcinogenesis have been described for all five organs in which humans develop cancer after exposure to inorganic arsenic (skin, lung, bladder, kidney, and liver, Table 1). Complete carcinogenesis by DMA has been achieved in rat bladder and mouse lung (Table 1). Of the four organs in which Yamamoto *et al.* (1995) originally described promotion of carcinogenesis following use of a multiple initiator protocol, experimental protocols using just a single initiator and subsequent promotion by DMA have been successfully developed for cancer of the rat bladder (Wanibuchi *et al.*, 1996) and liver (Wanibuchi *et al.*, 1997). A protocol that uses a single initiator and DMA promotion of carcinogenesis has also been developed for mouse lung (Yamanaka *et al.*, 1996). No carcinogenesis experiments have yet been reported with DMA administered to hamsters, dogs, or monkeys, species that do not develop tumors after inorganic arsenic administration.

Among mammals that methylate arsenic, humans are unusual because they excrete a relatively large amount of MMA (Vahter, 1994). Thus, human tissues may be exposed to much higher concentrations of pentavalent or trivalent MMA than are mice, rats, beagles, hamsters, or rabbits, for example (Vahter, 1994).

Trivalent arsenic species are capable of electronic interactions with biological molecules via the unshared 4 s electron pair. Pentavalent arsenic species lack this chemical reactivity due to this unshared pair of electrons. Several arsenic species form complexes with glutathione. Methylated trivalent arsenic species not only possess the reactive unshared electron pair but also contain one, two, or three methyl substituents that take the place of hydroxyl groups. These methyl groups could greatly change the properties of arsenic by increasing the hydrophobicity and decreasing the ionizability and negative charges due to hydroxyl group(s). Thus, likely candidates for the causal

TABLE 2
Arsenic Carcinogenesis in Transgenic Mice

Transgenic mice	Treatment protocol	Arsenic dose	Experimental finding	Reference
p53+/−	As(III), As(V), MMA, or DMA	50, 150, 1500, 1000	No tumors	Mass *et al.*, personal communication
p53+/−	DMA	50 ppm	Earlier tumors in p53+/−	Salim *et al.*, 1999
p53+/−	As(III) ALONE	50 ppm	No tumors	Popovicova *et al.*, 2000
	As(III) + *p*-CRESIDINE (0.2% IN DIET)	50 ppm	Increased bladder hyperplasia and carcinoma	
K6/ODC	DMA	10 ppm	8% Skin tumors (squamous papillomas)	Chen *et al.*, 2000
	DMA	100 ppm	22% Skin tumor (squamous papillomas)	
	As(III)	10 ppm	15% Skin tumors (squamous papillomas)	
K6/ODC	VEHICLE → DMA	3.6 mg	0.0 Skin tumors per mouse	Morikawa *et al.*, 2000
	DMBA → CONTROL	—	9.7 Skin tumors per mouse	
	DMBA → DMA	3.6 mg	19.4 Skin tumors per mouse	
Tg.AC	As(III)	200 ppm	No papillomas	Germolec *et al.*, 1998
	TPA	—	Some papillomas	
	As(III) and TPA	200 ppm	Increased number of papillomas	

carcinogenic species of arsenic would include MMA(III) in humans and DMA(III) in rats. The trimethylated compound TMA(III) in rats and arsenite in humans could also be involved in the carcinogenic process. However, the amount of evidence for TMA(III) as a carcinogenic arsenic species is considerably weaker than the evidence for arsenite, MMA(III), or DMA(III).

Transgenic Animal Models of Arsenic Carcinogenesis

$p53^{+/-}$ mice. The results of six different experiments performed in three types of transgenic mice are summarized in Table 2. No tumors that could be associated with arsenic exposure were found in one unpublished study of wild-type and p53-deficient mice (M. Mass, personal communication). These mice were exposed to either arsenite (50 ppm), arsenate (150 ppm), MMA (1500 ppm), or DMA (1000 ppm) in their drinking water for 1 year.

In an experiment with p53 heterozygous knockout and C57BL/6T wild-type male mice, DMA was given at 50 or 200 ppm in the drinking water for 80 weeks (Salim *et al.*, 1999). DMA exposure caused an increase in the total numbers of spontaneous tumors in wild-type mice and earlier induction of tumors in p53 knockout mice.

In a third experiment, $p53^{+/-}$ mice were exposed for 26 weeks to various combinations of arsenite, a choline-deficient diet, and *p*-cresidine, a known bladder carcinogen (Popovicova *et al.*, 2000; Moser *et al.*, 2000). Treatment with 50 ppm arsenite in the drinking water alone did not cause mouse tumors. In *p*-cresidine-exposed mice, coexposure to either arsenite or a choline-deficient diet enhanced the extent of bladder hyperplasia and the incidence of bladder carcinomas. Thus, although arsenite did not appear to be carcinogenic alone, with concomitant exposure to *p*-cresidine, arsenite appeared to be a cocarcinogen in $p53^{+/-}$ mice.

K6/ODC transgenic mice. ODC activity is induced in many epithelial tumors in both rodents and humans. ODC has been extensively studied in mouse skin and rat liver carcinogenesis systems and ODC induction is considered a biomarker for promotion (O'Brien *et al.*, 1976; Russell, 1985; Kitchin *et al.*, 1992). K6/ODC transgenic mice overexpress a truncated ODC protein in hair follicle keratinocytes. A bovine keratin IV (K6) promoter/regulatory region drives expression of ODC. In K6/ODC mice given either 10 ppm DMA, 100 ppm DMA, or 10 ppm arsenite in their drinking water for 5 months, 8, 22, or 15% of the mice developed skin squamous papillomas, respectively (Chen *et al.*, 2000). No tumors were observed in the control group of K6/ODC transgenic mice not exposed to arsenic and in nontransgenic littermates given 100 ppm DMA. These data in transgenic mice are one of the few observations of rodent tumors caused by inorganic arsenic exposure. No initiators of carcinogenesis were given to these K6/ODC mice. Instead these mice may function as highly promoted transgenic animals in which the initiating or progressing potential of a chemical is being assayed. Interestingly, at the same drinking water concentration of 10 ppm, arsenite and DMA had fairly similar tumor responses, 15 versus 8%, respectively. Thus, in this experimental system, trivalent arsenite was not more highly potent than pentavalent DMA, a surprising finding. Concentrations of arsenite above 10 ppm and DMA above 100 ppm were not tested. DMA concentrations as high as 400 ppm in A/J strain mice (Hayashi *et al.*, 1998) and 1000 ppm in mice (Yamanaka *et al.*, 2000) have been utilized in animal experimentation.

A two-stage mouse skin carcinogenesis experiment in K6/ODC mice was performed using the initiator 7,12-dimethylbenz[*a*]anthracene (DMBA, 50 μg, one application), 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 5 μg twice per week)

and DMA (3.6 mg twice per week) (Morikawa *et al.*, 2000). Twenty weeks after initiation, the average number of tumors per mouse was 20.7, 19.4, 9.7, and 0.0 in the four treatment groups of DMBA → TPA, DMBA → DMA, DMBA → control cream, and acetone vehicle → DMA, respectively. The DMBA → TPA treatment group responded with an increased tumor incidence about 2 weeks faster than the DMBA → DMA group. Microscopically, most tumors in the three responding treatment groups were squamous papillomas, with some squamous carcinomas with disorderly arrangement and atypical nuclei.

Tg.AC transgenic mice. One group has published results using Tg.AC female homozygous transgenic mice with the fetal ζ-globulin promoter fused to the v-Ha-*ras* structural gene (with mutations at codons 12 and 59) and linked to a simian virus 40 polyadenylylation/splice sequence (Gemolec *et al.*, 1998). Wild-type (FVB/N) or Tg.AC transgenic mice were exposed to combinations of TPA (given dermally twice per week for 2 weeks) and 200 ppm arsenite in the drinking water for up to 22 weeks. All mice were pretreated with arsenite for 4 weeks before TPA exposure. In wild-type mice exposed to arsenite and TPA together, no tumors were seen. In Tg.AC transgenic mice exposed to arsenite but no TPA, no tumors were observed. In Tg.AC transgenic mice, some skin papillomas were caused by TPA treatment alone. The number of mouse skin papillomas was increased if the dermal TPA treatment was combined with exposure to arsenite in the drinking water (Table 2). This research group has suggested arsenite was acting as a copromoter.

Summary. The key mutational events in arsenic carcinogenesis in humans are not yet known. Thus, at this time it is difficult to select or develop an appropriate transgenic animal model for arsenic carcinogenesis. None of the three transgenic mouse models tried so far have mimicked the pattern of human arsenic carcinogenesis with large elevations of skin, bladder, and lung carcinogenesis in the same animals.

In p53 transgenic animals exposed to 50 ppm arsenite or to 50 ppm DMA, no clear pattern of skin, lung, and bladder tumors was observed (Table 2). Thus, despite the importance of p53 in many human tumors and the known alterations in p53 in arsenic-induced human tumors, the p53 transgenic mouse system does not seem to be a good model for arsenic-induced carcinogenesis studies.

In Tg.AC transgenic mice, no skin tumors were seen following exposure to 200 ppm arsenite alone. A more promising transgenic model system is the K6/ODC transgenic mouse. Two different experimental groups have observed skin carcinogenesis in K6/ODC mice after arsenic exposure. In one case, either As(III) or DMA in the drinking water acted as a complete carcinogen (Chen *et al.*, 2000); in the other case topically applied DMA acted as a promoter (Morikawa *et al.*, 2000).

Overall, there is reason for limited optimism for the use of transgenic animals in the study of arsenic carcinogenesis. Both mouse skin (Chen *et al.*, 2000; Morikawa *et al.*, 2000) and bladder (Popovicova *et al.*, 2000) have shown some degree of carcinogenic response following arsenic exposure (Table 2). To date there are not any positive models of arsenic-induced lung carcinogenesis in transgenic animals. Once one or more key genetic events in the pathway of arsenic carcinogenesis in humans are known, use of transgenic animals may be much more fruitful.

METHYLATED METABOLITES OF ARSENIC

The generally held view of arsenic carcinogenesis in the past was that arsenite was the most likely cause of carcinogenesis and that methylation of arsenic species was a detoxification pathway. Some individuals had an almost Ptolemaic view that methylation of arsenic was of great importance in minimizing arsenic's toxicity and/or carcinogenicity. Other scientists, notably Aposhian (1989), have stressed the importance of arsenic's binding to proteins. Trivalent species are stronger protein-binding agents than are pentavalent arsenic species.

The present view of arsenic carcinogenesis is that there are many possible chemical forms of arsenic that may be causal in carcinogenesis and that methylation of arsenic may be a toxification, not a detoxification, pathway. In a relatively short period of time, evidence has accumulated in favor of this paradigm shift and a substantial change of scientific opinion has occurred. Much of the present interest centers on the trivalent methylated species of arsenic–MMA(III), DMA(III), and to a lesser extent TMA(III)–which may be formed by reductive processes from pentavalent arsenicals. TMA(III) may exist in higher concentrations in rat tissues than in human tissues.

MMA(III)

MMA(III) has been found in urine of humans exposed to arsenic without (Aposhian *et al.*, 2000a) and with concomitant treatment with chelators (Aposhian *et al.*, 2000b). MMA(III) is an excellent choice as a cause of arsenic carcinogenesis because humans excrete much more MMA than any other species, thus providing a reason why humans are found to be unusually sensitive to arsenic-induced carcinogenesis. Some of the biological activities that MMA(III) is known to possess in various experimental systems include enzyme inhibition (Lin *et al.*,1999; Styblo *et al.*, 1997), cell toxicity (Petrick *et al.*, 2000), and genotoxicity (Mass *et al.*, 2001). This certainly is an impressive list of biological activities for a methylated arsenic metabolite formerly thought to be a "detoxified" form of arsenic.

DMA(III)

DMA(III), as well as MMA(III), has been demonstrated in human urine of arsenic-exposed humans administered 2,3-dimercapto-1-propane sulfonate (a chelator) (Le *et al.*, 2000). In a study of hamsters given arsenate (Sampayo-Reyes *et al.*,

2000), substantial hepatic concentrations of trivalent MMA and DMA were found in addition to the expected pentavalent MMA and DMA. MMA reductase activity was fairly high in hamster bladder tissue (Sampayo-Reyes *et al.*, 2000). The capacity to separate the trivalent forms of the methylated arsenic species from the pentavalent forms has just recently been developed (Del Razo *et al.*, 2000; Sampayo-Reyes *et al.*, 2000; Le *et al.*, 2000). Future research will probably give us a much better view of the relative tissue ratios of trivalent to pentavalent arsenic chemical forms for each of the methylated species. Few data are currently available on the tissue concentrations of trivalent methylated species. In rats, the oral LD50 is approximately 100 for arsenate, 41 for arsenite, 961 for MMA, and 644 mg/kg for DMA (Brown *et al.*, 1997). The high LD50 values for administered pentavalent MMA and DMA suggest that in rats only a small fraction of these methylated arsenic forms is both trivalent and biologically active at any given time. After pentavalent methylated arsenicals have generated a certain amount of trivalent methylated arsenicals by reductive processes, protein binding of trivalent methylated arsenicals to noncritical sulfhydryl targets may protect the organism from trivalent methylated arsenicals binding to critical sites that cause toxicity or carcinogenicity. Otherwise, biologically active trivalent methylated arsenicals might have produced death in the experimental animals at much lower oral doses than 644 mg/kg of DMA or 961 mg/kg of MMA. There have been many animal models of arsenic carcinogenesis developed in which pentavalent DMA was given to rats or mice (Tables 1 and 2). In these models, the cancers are likely due to the trivalent species of DMA(III) or TMA(III) or alternatively to the pentavalent species DMA or TMAO. As an organism's cells are exposed to a whole range of arsenic metabolites, it may be very naive to think of just one causative arsenic species. Similarly, it may be naive to think of just one causative mode of action of carcinogenesis for arsenic. For example, arsenic carcinogenesis might arise from the combination of chromosomal abnormalities, oxidative stress, and the augmentation of growth factors causing cell proliferation and eventually promotion of carcinogenesis. Some of the biological activities that DMA(III) is known to possess in various experimental systems include enzyme inhibition (Lin *et al.*, 1999; Styblo *et al.*, 1997) cell toxicity (Petrick *et al.*, 2000), genotoxicity, and clastogenicity (Mass *et al.*, 2001).

If DMA(III) produced from pentavalent DMA is the causative chemical of arsenic carcinogenesis, it is wise to remember that any animal experiments performed with administered DMA(III) may significantly differ in the tissue levels of DMA(III) achieved and in the pharmacokinetics of the administered DMA(III) compared to that of DMA(III) produced from administered pentavalent DMA.

At physiological pH, DNA is negatively charged from the phosphate groups. The bases have a substantial number of electronegative atoms, such as oxygen and nitrogen, which are frequently adducted by chemicals. Many arsenic chemical forms have a substantial negative charge at physiological pH, so these negatively charged arsenic forms would not be expected to easily interact with DNA because of electrostatic repulsion. When arsenic is successively methylated, the ionizable hydroxy groups are replaced by uncharged methyl groups. Thus, a molecule of DMA(III) may be able to directly interact with DNA far more easily than trivalent arsenite.

TMA(III)

TMA(III) may be produced from TMAO by reduction. Data from one group suggest that only rats may have relatively high levels of TMAO in their urine. Rats are also the most responsive animal model for arsenic carcinogenesis yet found. A molecule of TMA(III) possesses no ionizable hydroxyl groups to limit the ability of this trivalent arsenic species to interact with DNA.

Summary

The data from rodent animal models that respond to administered pentavalent DMA have been criticized for the high concentrations of pentavalent DMA that are required for promotion of carcinogenesis or for complete carcinogenesis (Table 1). It may be that the trivalent compound DMA(III) is actually the active carcinogenic species and that pentavalent DMA is entirely or largely inactive. Thus, the observation that only high drinking water or dietary concentrations of pentavalent DMA are active in experimental systems of carcinogenesis is exactly as it should be. Pentavalent DMA is a poor delivery system for DMA(III) in rats. Similarly, inorganic arsenic exposure is a poor delivery system for either MMA(III) or DMA(III) in humans. Nonetheless, in both humans and rats, either MMA(III) or DMA(III) could be the cause of arsenic carcinogenesis. These trivalent methylated arsenicals should be vigorously investigated for biological and carcinogenic activity.

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REFERENCES

Abernathy, C. O., Lui, Y. P., Longfellow, D., Aposhian, H. V., Beck, B., Fowler, B., Goyer, R., Menzer, R., Rossman, T., Thompson, C., and Waalkes, M. (1999). Arsenic: Health effects, mechanisms of actions and research issues. *Environ. Health Perspect.* **107,** 593–597.

Ahmad, S., Kitchin, K. T., and Cullen, W. R. (2000). Arsenic species that cause release of iron from ferritin and generation of activated oxygen. *Arch. Biochem. Biophys.* **382**, 195–202.

Arnold, L. L., Cano, M., St. John, M., Eldna, M., van Gemert, M., and Cohen, S. M. (1999). Effects of dietary dimethylarseinic acid on the urine and urothelium of rats. *Carcinogenesis* **20,** 2171–2179.

Aposhian, H. V. (1989). Biochemical toxicology of arsenic. In *Reviews in Biochemical Toxicology* (E. Hodgson, J Bend, and R. Philpot, Ed.), Vol. 10, pp. 265–300. Elsevier, New York.

Aposhian, H. V., Gurzau, E. S., Le, X. C., Gurzau, A., Healy, S. M., Lu, X., Ma, M., Yip, Li., Zakharyan, R. A, Maiorino, R. M., Dart, R. C., Tircus, M. G., Gonzalez-Ramirez, D., Morgan, D. L. Avram, D., and Aposhian, M. M. (2000a). Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem. Res. Toxicol.* **13,** 693–697.

Aposhian, H. V., Zheng, B., Aposhian, M. M., Le, X. C., Cebrian, M. E., Cullen, W., Zakharyan, R. A., Ma, M., Dart, R. C., Cheng, Z., Andrewes, P., Yip, Li., O'Malley, G. F., Maiorino, R. M., Van Voorhies, W., Healy, S. M., and Titcomb, A. (2000b). DMPS-arsenic challenge test. II. Modulation of arsenic species, including monomethylarsonous acid(MMA(III)), excreted in human urine. *Toxicol. Appl. Pharmacol.* **165,** 74–83.

Beckman, G., Beckman, L., and Nordenson, I. (1977). Chromosome aberrations in workers exposed to arsenic. *Environ. Health Perspect.* **19,** 145–146.

Boonchai, W., Walsh, M., Cummings, M., and Chenevix-Trench, G. (2000). Expression of p53 in arsenic-related and sporadic basal cell carcinoma. *Arch. Dermatol.* **136,** 195–198.

Brown, J., and Kitchin, K. T. (1996). Arsenite, but not cadmium, induces ornithine decarboxylase and heme oxygenase activity in rat liver: Relevance to arsenic carcinogenesis. *Cancer Lett.* **98,** 227–231.

Brown, J. L., Kitchin K. T., and George, M. (1997). Dimethylarsinic acid treatment alters six different rat biochemical parameters: Relevance to arsenic carcinogenesis. *Teratog. Carcinog. Mutagen.* **17,** 71–84.

Castren, K., Ranki, A., Welsh, J. A., and Vahakangas, K. H. (1998). Infrequent p53 mutations in arsenic-related skin lesions. *Oncol. Res.* **10,** 475–482.

Chen Y., Megosh, L. C., Gilmour, S. K., Sawicki, J. A., and O'Brien, T. G. (2000). K6/ODC transgenic mice as a sensitive model for carcinogen identification. *Toxicol. Lett.* **116**, 27–35.

Cherney, B. W., McBride, O. W., Chen, D., Alkhatib, H., Bhakia, K., Hensen, P., and Smulson, M. E. (1987). cDNA sequence, protein structure, and chromosomal location of the human gene for poly (ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA* **84,** 8370–8374.

Clayson, D. B., and Kitchin, K. T. (1999). Interspecies differences in response to chemical carcinogens. In *Carcinogenicity: Testing Predicting, and Interpreting Chemical Effects* (K. Kitchin, Ed.), pp. 837–880. Dekker, New York.

Cohen, S. M., Yamamoto, S., Cano, M., and Arnold, L. L. (2001). Urothelial cytotoxicity and regeneration induced by dimethylarsinic acid in rats. *Toxicol. Sci.* **59,** 68–74.

Del Razo, L. M., Styblo, M., and Thomas, D. J. (2000). Determination of trivalent methylated arsenic species in water, cultured rat hepatocytes, and human urine. *4th International Conference on Arsenic Exposure and Health Effects* Poster 10.

EPA (1981). *Methane Arsonic Acid (MMA): 2-Year Chronic/Onogenic Rat Feeding Study.* Memorandum from W. Dykstra, Toxicology Branch, Health Effects Division to R. Mountfort, Registration Division. May 13, U.S. EPA Office of Pesticides and Toxic Substances, Washington, D.C.

EPA (1994). *Carcinogenicity Peer Review of Cacodylic Acid.* Memorandum from S. Malish and E. Rinde to C. Giles-Parker and J. Elllenberger. U.S. EPA, Office of Pesticides and Toxic substances, Washington, D. C.

Germolec, D. R., Spalding, T., Boorman, G. A., Wilmer, J. L., Yoshida, T., Simeonova, P. D., Bruccoleri, A., Kayama, F., Gaido, K., Tennant, R., Burleson, F., Dong, W., Lang, R. W., and Luster, M. I. (1997). Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors. *Mutat. Res.* **386,** 209–218.

Germolec, D. R., Spalding, T., Yu, H. S., Chen, G. S., Simeonova, P. D., Humble, M. C., Bruccoleri, A., Boorman, G. A., Foley, J. F., Yosida, T., and Luster, M. I. (1998). Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. *Am. J. Pathol.* **153,** 1775–1785.

Gonsebatt, M. E., Vega, L., Salazar, A. M., Montero, R., Guzman, P., Blas, J., DelRazo, L. M., Garcia-Vargas, G., Albores, A., Cebrian, M. E., Kelsh, M., and Ostrosky-Wegman, P. (1997). Cytogenic effects in human exposure to arsenic. *Mutat. Res.* **386,** 219–228.

Gurr, J., Liu, F., Lynn, S., and Jan K. (1998). Calcium-dependent nitric oxide production is involved in arsenite-induced micronuclei. *Mutat. Res.* **416,** 137–148.

Hamadeh H. K., Vargas, M., Lee E., and Menzel, D. B. (1999). Arsenic disrupts cellular levels of p53 and mdm2: A potential mechanism of carcinogenesis. *Biochem. Biophys. Res. Commun.* **263,** 446–449.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100,** 57–70.

Hayashi, H., Kanisawa, M., Yamanaka, K., Ito, T., Udaka, N., Ohji, H., Okudela, K., Okada, S., and Kitamura, H. (1998). Dimethylarsinic acid, a main metabolite of inorganic arsenics, has tumorigenicity and progression effects in the pulmonary tumors of A/J mice. *Cancer Lett.* **125,** 83–88.

Hsu, C. H., Yang, S. A., Wang, J. Y., and Lin, S. R. (1999). Mutational spectrum of p53 gene in arsenic-related skin cancers from the blackfoot disease endemic area of Taiwan. *Br. J. Cancer* **80,** 1080–1086.

Hu, Y., Su, L., and Snow, E. T. (1998). Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition on DNA repair enzmes. *Mutat. Res.* **408,** 203–218.

Husain, I., Van Houten, B., Thomas, D. C., and Sancar, A. (1986). Sequences of *Escherichia coli* uvrA gene and the protein reveal two potential ATP binding sites. *J. Biol. Chem.* **261,** 4895–4901.

Jones, J. S., Weber, S., and Prakash, L. (1988). The *Saccharomyces cerevisiae* RAD18 gene encodes a protein that contains potential zinc finger domains for nucleic acid binding and a putative nucleotide binding sequence. *Nucleic Acids Res.* **16,** 7119–7131.

Katakura, K., and Chang, K. P. (1989). H DNA amplification in *Leishmania* resistant to both arsenite and methotrexate. *Mol. Biochem. Parisitol.* **34,** 189–192.

Kenyon, E. M., and Hughes M. F. (2001). A concise review of the toxicology and carcinogenicity of dimethylarsinic acid. *Toxicology* **160,** 227–236.

Kitchin, K., Brown, J., and Kulkarni, A. (1992). Predictive assay for rodent carcinogenicity using in vivo biochemical parameters: Operational characteristics and complementarity. *Mutat. Res.* **266,** 253–272.

Kitchin, K. T., Del Razo, L. M., Brown, J. L., Anderson, W. L., and Kenyon, E. M. (1999). An integrated pharmacokinetic and pharmacodynamic study of arsenite action. 1. Heme oxygenase induction in rats. *Teratog. Carcinogen. Mutag.* **19,** 385–402.

Le, X. C., Lu, X., Ma, M., Cullen, W. R., Aposhian, H. V., and Zheng, B. (2000). Speciation of key arsenic metabolic intemediates in human urine. *Anal. Chem.* **72,** 5172–5177.

Lee, T. C., Tanaka, N., Lamb, P. W., Gilmer, T. M., and Barrett, J. C. (1988). Induction of gene amplification by arsenic. *Science* **241,** 79–81.

Lee-Chen, S. F., Wang, M. C., Yu, C. T., Wu, D. R., and Jan, K. Y. (1993). Nickel chloride inhibits the DNA repair of UV-treated but not methyl methanesulfonate-treated Chinese hamster ovary cells. *Biol. Trace Elem. Res.* **37,** 39–50.

Li, J. H., and Rossman, T. G. (1989). Mechanism of comutagenesis of sodium arsenite with *N*-methyl-*N*-nitrosourea. *Biol. Trace Elem.* **21,** 373–381.

Life Sciences Research Israel (1989). *Cacodylic Acid: Combined Chronic Feeding and Oncogenicity Study in the Rat.* Study PAL/010/CAC. [MRID. 418621–01, HED Documents. 009391, 010550].

Lin, S., Cullen, W. R., and Thomas, D. J. (1999). Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. *Chem. Res. Toxicol.* **12,** 924–930.

Liu, J., Kadiiska, M., Liu, Y., Qu, W., Mason, R. P., and Waalkes, M. P. (2000). Acute arsenic-induced free radical production and oxidative stress-related gene expression in mice. *Toxicologist* **54**, 280–281.

Mass, M. J., Tennant, A., Roop, B., Kundu, K., Brock, K., Kligerman, A., Demarini, D., Wang, C., Cullen, W., Thomas, D., and Styblo, M. (2001). Methylated arsenic(III) species react directly with DNA and are potential proximate or ultimate genotoxic forms of arsenic. *Toxicologist* **60,** 358.

Mass, M. J., and Wang, L. (1997). Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: A model for a mechanism of carcinogenesis. *Mutat. Res.* **386,** 263–277.

Matsui, M., Nishigori, C., Toyokuni, S., Takada, J., Mitsuhiko, A., Ishikawa, M., Imamura, S., and Miyachi, Y. (1999). The role of oxidative DNA damage in human arsenic carcinogenesis: Detection of 8-hydroxy-2′-deoxyguanosine in arsenic-related Bowen's disease. *J. Invest. Dermatol.* **113,** 26–31.

Moolgavkar, S. H. (1986). Carcinogenesis modeling: From molecular biology to epidemiology. *Annu. Rev. Public Health* **7,** 151–169.

Morikawa, T., Wanibuchi H., Morimura K., Ogawa M., and Fukushima, S. (2000). Promotion of skin carcinogenesis by dimethylarsinic acid in keratin (K6)/ODC transgenic mice. *Jpn. J. Cancer Res.* **91,** 579–581.

Moser, G., Goldsworthy T., and Tice R. (2000). *Sodium Arsenite Studies in p53$^{+/-}$ Mice*. American Water Works Foundation, Denver, CO.

National Research Council (1999). Mechanisms of toxicity. In *Arsenic in Drinking Water*. pp. 194–196. National Academy Press, Washington, D.C.

Nordenson, I., and Beckman, L. (1991). Is genotoxic effect of arsenic mediated by oxygen free radicals? *Hum. Hered.* **41,** 71–73.

Nordenson, I., Beckman, G., Beckman, L., and Nordstrom, S. (1978). Occupational and environmental risks in and around a smelter in Northern Sweden. II. Chromosomal abberations in workers exposed to arsenic. *Hereditas* **88,** 263–267.

O'Brien, T. G. (1976). The induction of ornithine decarboxylase as an early possibly obligatory event in mouse skin carcinogenesis. *Cancer Res.* **36,** 2644–2653.

Petres, J., Baron, D., and Hagedorn, M. (1977). Effects of arsenic cell metabolism and cell proliferation: Cytogenetic and biochemical studies. *Environ. Health Perspect.* **19**, 223–227.

Petrick, J. S., Ayala-Fierro, F., Cullen W. R., Carter, D. E., and Aposhian, H. V. (2000). Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes. *Toxicol. Appl. Pharmacol.* **163,** 203–207.

Popovicova, J., Moser, G. J., Goldsworthy, T. L., and Tice, R. R. (2000). Carcinogenicity and co-carcinogenicity of sodium arsenite in p53$^{+/-}$ male mice. *Toxicologist* **54**, 134.

Russell, D. H. (1985). Ornithine decarboxylase: A key regulatory enzyme in normal and neoplastic growth. *Drug Metab. Rev.* **16,** 1–88.

Salazar, A. M., Ostrosky-Wegman, P., Menendez, D., Miranda, E., Garcia-Carranca, A., and Rojas, E. (1997). Induction of p53 protein expression by sodium arsenite. *Mutat. Res.* **381,** 259–265.

Salim, E. I., Wanibuchi, H., Yamamoto, S., Morimura, K., and Fukushima, S. (1999). Carcinogenicity of dimethylarsinic acid (DMAA) in p53 knockout and wild type C57BL/6T mice. *Proc. Am. Assoc. Cancer Res.* **40,** 2335. [Abstract]

Sampayo-Reyes, A., Zakharyan, R. A., Healy, S. H., and Aposhian, H. V. (2000). Monomethylarsonic acid reductase and monomethyarsonous acid in hamster tissue. *Chem. Res. Toxicol*. **13,** 1181–1186.

Simeonova, P. P., and Luster, M. I. (2000). Mechanisms of arsenic carcinogenicity: Genetic or epigenetic mechanisms? *J. Environ. Pathol. Toxicol. Oncol.* **19,** 281–286.

Styblo, M., Serves, S. V., Cullen, W. R., and Thomas, D. J. (1997). Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. *Chem. Res. Toxicol.* **10,** 27–33.

Tanaka, K., Miura, N., Satokata, I., Miyamoto, I., Yoshida, M. C., Satoh, Y., Kondo, S., Vasui, A., Okayama, H., and Okada, Y. (1990). Analysis of a human DNA excision repair gene in group A xeroderma pigmentosum and containing a zinc finger domain. *Nature* **348,** 73–76.

Vahter, M. (1994). Species differences in the metabolism of arsenic compounds. *Appl. Organomet. Chem.* **8,** 175–182.

Vega, L., Styblo, M., Patterson, R., Cullen, W., Wang, C., and Germolec, D. (2001). Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in normal human epidermal keratinocytes. *Toxicol. Appl. Pharmacol.* (in press).

Wanibuchi, H., and Fukushima, S. (2000). Carcinogenicity of an organic arsenic, dimethylarsenic acid in animals. *XXIII International Congress of The International Academy of Pathology and 14th World Congress of Academic and Environmental Pathology*, Nagoya, Japan.

Wanibuchi, H., Hori, T., Meenakshi, V., Ichihara, T., Yamamoto, S., Yano, Y., Otani, S., Nakae, D., Konishi, Y., and Fukushima, S. (1997). Promotion of rat hepatocarcinogensesis by dimethylarsinic acid as assessed in rat *in vivo* models: A review. *Mutat. Res.* **386,** 353–361.

Wanibuchi, H., Wei, M., Yamamoto, S., Li, W., and Fukushima, S. (2000). Carcinogenicity of an organic arsenical, dimethylarsenic acid and related arsenicals in rat urinary bladder. *Proc. Am. Assoc. Cancer Res.* **40,** 2309. [Abstract]

Wanibuchi, H., Yamamoto, S., Chen, H., Yoshida, K., Endo, G., Hori, T., and Fukushima, S. (1996). Promoting effects of dimethylarsinic acid of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. *Carcinogenesis* **17,** 2435–2439.

Warner, M. L., Moore, L. E., Smith, M. T., Kalman, D. A., Fanning, E., and Smith, A. H. (1994). Increased micronuclei in exfoliated bladder cells of individuals who chronically ingest arsenic-contaminated water in Nevada. *Cancer Epid. Biomarkers Prevent.* **3,** 583–590.

Wei, M., Wanibuchi, H., Yamamoto, S., Li, W., and Fukushima, S. (1999). Urinary bladder carcinogenicity of dimethylarsinic acid in male F344 rats. *Carcinogenesis* **20,** 1873–1876.

Woloson, D. (1990). *Gene amplification in SV40 transformed human keratinocytes.* Ph.D. Thesis, New York University Medical School, New York.

Yager, J. W., and Wiencke, J. K. (1997). Inhibition of poly(ADP-ribose)polymerase by arsenite. *Mutat. Res.* **386,** 345–351.

Yamamoto, S., Konishi, Y., Matsuda, T., Murai, T., Shibata, M., Matsui-Yuasa, I., Otani, S., Kuroda, K., Endo, G., and Fukushima, S. (1995). Cancer induction by an organic arsenic compound, dimethylarsinic acid (cacodylic acid), in F344/DuCrj rats after pretreatment with five carcinogens. *Cancer Res.* **55,** 1271–1275.

Yamanaka, K., Hoshino, M., Okanoto, M., Sawamura, R., Hasegawa, A., and Okada, S. (1990). Induction of DNA damage by dimethylarsine, a metabolite of inorganic arsenics, is for the major part likely due to its peroxyl radical. *Biochem. Biophys. Res. Commun.* **168,** 58–64.

Yamanaka, K., Katsumata, K., Ikuma, K., Hasegawa, A., Nakano, M., and Okada, S. (2000). The role of orally administered dimethylarsinic acid, a main metabolite of inorganic arsenics, in the promotion and progression of UVB-induced skin tumorigenesis in hairless mice. *Cancer Lett.* **152,** 79–85.

Yamanaka, K., Mizoi, M., Kato, K., Hasegawa, A., Nakano, M., and Okada, S. (2001). Oral administration of dimethylarsinic acid, a main metabolite of inorganic arsenics, promote skin tumorigenesis in mice. *Biol. Pharm. Bull. Japan.* **24**.

Yamanaka, K., Ohtsubo, K., Hasegawa, A., Hayashi, H., Ohji, H., Kanisawa, M., and Okada, S. (1996). Exposure to dimethylarsinic acid, a main metabolite of inorganic arsenics, strongly promotes tumorigenesis initiated by 4-nitroquinoline-1-oxide in the lungs of mice. *Carcinogenesis* **17,** 767–770.

Yamanaka, K., and Okada, S. (1994). Induction of lung-specific DNA damage by metabolically methylated arsenics via the production of free radicals. *Environ. Health Perspect.* **102,** 37–40.

Zhao, C. Q., Young, M. R., Diwan, B. A., Coogan, T. P., and Waalkes, M. P. (1997). Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proc. Natl. Acad. Sci. USA* **94,** 10907–10912.

Zhong, C. X., Wang, L., and Mass, M. J. (2001). Differentially methylated DNA sequences associated with exposure to arsenite in cultures of human cells identified by methylation-sensitive arbitrarily-primed PCR. (submitted for publication).