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Review

Inorganic arsenic: A non-genotoxic carcinogen

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ABSTRACT

Inorganic arsenic induces a variety of toxicities including cancer. The mode of action for cancer and non-cancer effects involves the metabolic generation of trivalent arsenicals and their reaction with sulfhydryl groups within critical proteins in various cell types which leads to the biological response. In epithelial cells, the response is cell death with consequent regenerative proliferation. If this continues for a long period of time, it can result in an increased risk of cancer. Arsenicals do not react with DNA. There is evidence for indirect genotoxicity in various *in vitro* and *in vivo* systems, but these involve exposures at cytotoxic concentrations and are not the basis for cancer development. The resulting markers of genotoxicity could readily be due to the cytotoxicity rather than an effect on the DNA itself. Evidence for genotoxicity in humans has involved detection of chromosomal aberrations, sister chromatid exchanges in lymphocytes and micronucleus formation in lymphocytes, buccal mucosal cells, and exfoliated urothelial cells in the urine. Numerous difficulties have been identified in the interpretation of such results, including inadequate assessment of exposure to arsenic, measurement of micronuclei, and potential confounding factors such as tobacco exposure, folate deficiency, and others. Overall, the data strongly supports a non-linear dose response for the effects of inorganic arsenic. In various *in vitro* and *in vivo* models and in human epidemiology studies there appears to be a threshold for biological responses, including cancer.

Introduction

Arsenic has been known as a toxic, poisonous substance for many centuries (Cullen, 2008). Its possible relationship to cancer was first described more than a century ago in individuals being administered various solutions as potential therapeutic agents. Its association with skin changes (arseniasis) and ultimately cancer (basal cell and squamous cell carcinomas) was confirmed by observations in patients given an arsenical for treatment of syphilis that had been developed by Ehrlich, for which he received the Nobel Prize (Cullen, 2008; Neubauer, 1947). Beginning with the seminal publication by Chen et al. (1985) in the early 1980s, an awareness developed of a relationship between high exposure to inorganic arsenic in the drinking water and cancer of the urinary bladder. Exposure to inorganic arsenic in various mining occupations led to the discovery that it also could produce cancer of the lung, which was confirmed later as also arising from oral exposure (NRC, 1999, 2001). Subsequently, other tumors have been identified as being associated with inorganic arsenic such as tumors of the

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kidney and liver (Cohen et al., 2013). However, recently it has been demonstrated that the kidney tumors were actually those arising from the kidney pelvis, not the renal parenchyma, and are urothelial tumors similar to those in the urinary bladder (Ferreccio et al., 2013). The kidney pelvis is lined by the same type of epithelium, the urothelium, as the urinary bladder. Thus, the kidney pelvis tumors are likely due to a similar mechanism that is involved with the urinary bladder. The association with liver cancer has been recently described in various epidemiology studies, although the evidence is not as strong as for the skin, urothelium, and lung (Cohen et al., 2013).

The studies that demonstrated a relationship of exposure to inorganic arsenic with various types of cancer involved exposure to very high levels, either in the drinking water or the air (by inhalation). Inorganic arsenic exposure by inhalation is related to certain mining occupations. This exposure has been significantly reduced due to protective measures that have been implemented (IARC, 2012). However, exposure to inorganic arsenic in the drinking water in some parts of the world remains at extremely high levels, such as in Taiwan, China, Bangladesh, India, Chile, Argentina, and Mexico (IARC, 2012; Cohen et al., 2013). Most of the world, including the United States, Europe, and most of Asia has exposures in the drinking water at substantially lower levels than those that have been described in association with various types of cancer (NRC, 2001; IARC, 2012; Cohen et al., 2013).

The dose response for inorganic arsenic has not been clearly delineated in human populations. The concern is that it extends to low exposures rather than involving only high exposures. Whether exposure to inorganic arsenic at lower levels (less than 10 parts per billion in the drinking water) might be associated with an increased risk of cancer can only be determined by an understanding of the mode of action by which inorganic arsenic induces cancer. Abernathy et al. (1996) nearly two decades ago suggested that inorganic arsenic is a threshold carcinogen. Based on our understanding of the mode of action involved with arsenical induction of various types of cancers, the scientific evidence involving investigations *in vitro*, *in vivo* and in epidemiology, now strongly supports such a conclusion (Cohen et al., 2013). The evidence for this will be presented in this manuscript.

1. Effect of cancer mode of action on dose–response relationship

Cancer is due to multiple errors in DNA that can either be inherited or occur during DNA replication (Cohen and Arnold, 2011). The multiple genetic errors must be present in a single cell for cancer to develop, since cancer is a clonal disease. Although known for many decades, it is also now well-accepted that cancers arise from pluripotential cells in tissues, that are commonly referred to as tissue stem cells (Armitage and Doll, 1954; Moolgavkar and Knudson, 1981; Greenfield et al., 1984; Cohen and Ellwein, 1990; Cohen and Arnold, 2011). The errors can occur during DNA replication either by direct damage to the DNA (DNA reactive, genotoxic) or by "spontaneous" errors that occur during DNA replication. If the number of DNA replications is increased by environmental stimuli, the number of these spontaneous errors can be increased. Substances that directly damage DNA are referred to as DNA reactive carcinogens. A broader term for agents that damage DNA is genotoxic carcinogens. Substances that increase the risk of cancer by increasing the number of cell replications without direct damage to DNA are referred to as non-genotoxic or non-DNA reactive carcinogens.

It has been assumed for several decades that DNA reactive carcinogens do not involve a threshold, although there is some evidence that thresholds might also be involved in such instances (Doak et al., 2007). Nevertheless, if genotoxicity is produced indirectly rather than by direct interaction with DNA, or if cancer is induced by a non-genotoxic mechanism, a threshold response is involved.

Genotoxicity can be produced either by direct interaction of the agent with DNA (DNA reactive) or by indirect effects that produce errors in DNA (Cohen and Arnold, 2011). These indirect effects can involve interactions with a number of proteins involved in the mitotic process, such as tubulin, or processes that lead to micronucleus formation or chromosomal aberrations. In addition, inhibition of DNA repair enzymes could also lead to an indirect genotoxic process (Cohen and Arnold, 2011; Cohen et al., 2013).

Indirect effects on the DNA have also been postulated to occur either by oxidative damage or by peroxidation. Although examples have been identified in certain *in vitro* (Gentry et al., 2010; Yager et al., 2013) and *in vivo* animal models (Wei et al., 2005), it remains unclear whether oxidative stress itself can actually lead to an increase in cancer (Snow et al., 2005; Cohen et al., 2013; Gentry et al., 2014a, 2014b; Scudellari, 2015) (For a more detailed discussion, see below).

Increased cell replications in the stem cell population can occur either by increasing cell births or decreasing cell deaths (which increases the number of cells) (Cohen and Ellwein, 1991; Cohen and Arnold, 2011). It is not the rate of cell replication that is critical but the total number of replications. Thus, if the number of cells is increased by decreasing cell death, even if the rate of replication is at normal levels there will be an increase in DNA replications. This appears to be particularly critical in tissues in which there already is a high replication rate, such as colon, skin, or bone marrow. Increased cell births can be produced either be direct mitogenesis, which usually involves certain hormones or growth factors, or by cytotoxicity with consequent regeneration. In epithelia such as the skin, bladder, or lung, which have cell layers, the increase in cell number is evident in the form of hyperplasia. Most commonly hyperplasia involves not only an increase in the cell number but an increase in the replication rate.

2. Arsenic metabolism

To better understand the mode of action involved with inorganic arsenic-induced cancer (Fig. 1), a basic understanding of the metabolism of inorganic arsenic is necessary. Inorganic arsenic undergoes a series of reductions of the +5 oxidative state to the +3 oxidative state followed by oxidative methylation (Thomas, 2007; Cullen, 2008; Cohen et al., 2013). The sequence appears to involve inorganic arsenate (iAs^{+V}) being reduced to arsenite (iAs^{+III}), then methylated to monomethylarsonic acid (MMA^{V}), which is reduced to monomethylarsonous acid (MMA^{III}) and then methylated to dimethylarsinic

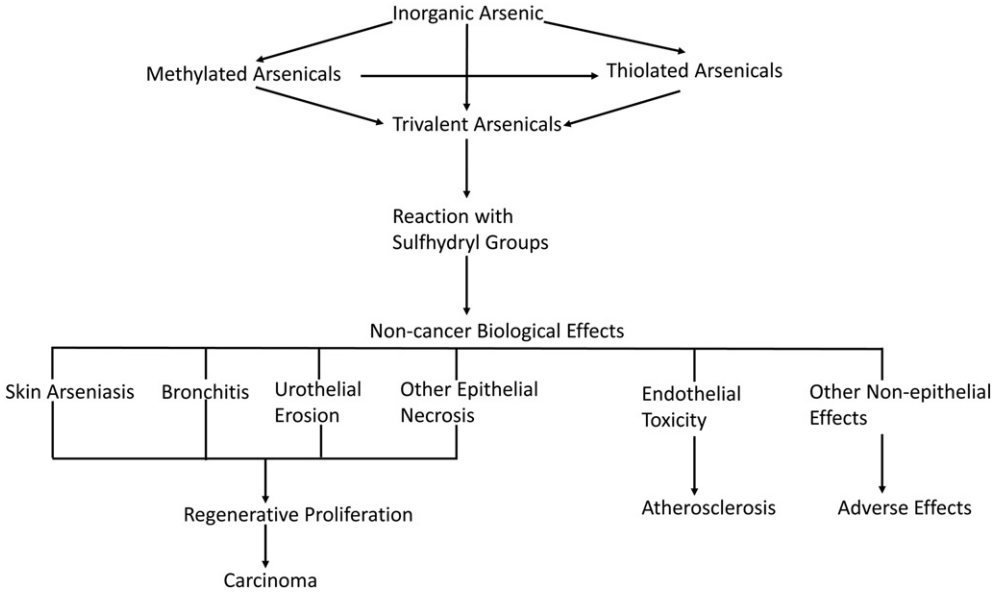

Fig. 1 – The metabolism of inorganic arsenic involves methylation. In addition, inorganic arsenic or the methylated arsenical (pentavalent or trivalent) can be thiolated. Thiolated arsenicals can rapidly enter cells and be converted to trivalent oxygen-containing arsenicals. The trivalent arsenicals can covalently bind free sulfhydryl groups, either in small molecules like glutathione or in proteins. Depending on the animal species and the cell type, different proteins will be affected. Once a threshold is exceeded, the functions of the proteins are altered, leading to biologic effects. If in epithelial tissues, cell death with regenerative proliferation will occur, ultimately leading to carcinoma. In other tissues, other effects occur with different adverse consequences, such as atherosclerosis.

acid (DMA^V). DMA^V can be reduced to dimethylarsinous acid (DMA^{III}) and further methylated to trimethylarsine oxide (TMAO). Except under unusual circumstances, formation of the corresponding arsines does not occur in mammalian systems. Although the conversion of DMA^V to TMAO commonly occurs in various species, especially in rodents, this occurs in humans only when exposures to inorganic arsenic are extremely high (potentially lethal) (Cohen et al., 2013). The enzyme involved with arsenic methylation is arsenic +3 methyltransferase (As3mt) (Thomas, 2007). In rodents, iAs^V, MMA^V and DMA^V are excellent substrates for this enzyme so that TMAO readily forms. In contrast, DMA^V in humans is a poor substrate for this enzyme, requiring exceedingly high concentrations for the additional methyl group to be added (Thomas, 2007). Under usual exposures to inorganic arsenic in the environment, even as high as 1000 ppb and higher in the drinking water, TMAO is rarely observed in humans (Cohen et al., 2013).

It has been demonstrated in a variety of cell systems that the trivalent forms of arsenic are the toxic forms (Cohen et al., 2013). Inorganic arsenate is less toxic than arsenite, and it has been suggested that it is toxic because of its reduction to arsenite. Trivalent arsenicals (iAs^{III}, MMA^{III}, and DMA^{III}) are highly toxic to cells at concentrations ranging from 0.1 μmol/L to less than 10 μmol/L (Gentry et al., 2010; Cohen et al., 2013; Dodmane et al., 2013). In general, concentrations greater than 10 μmol/L are lethal to all cell types. At concentrations 0.1 μmol/L and below, the changes in cells are adaptive in nature, primarily dealing with the metabolism of the compound and its excretion, rather than producing an adverse effect (Gentry et al., 2010). A cellular threshold appears to involve concentrations of 0.1 μmol/L and above, generally at levels higher than this. For environmental exposures to inorganic arsenic to produce any type of toxicity, tissue concentrations of 0.1 μmol/L or higher must be generated (Cohen et al., 2013). At concentrations lower than that, no adverse reaction occurs. In contrast, MMA^V, DMA^V, and TMAO have very low toxicity to cells, with toxicity occurring generally at millimolar concentrations and above Dodmane et al., 2013).

In the past 10 years, numerous thiolated arsenicals have been identified, the corresponding mono or dithiol analogs of the oxygen-containing arsenicals (Thomas, 2010). For oxygen-containing arsenicals, inorganic arsenic is readily transported across cell membranes, although the transporter for arsenite is different than for arsenate (Cohen et al., 2013). The transporters for arsenate tend to be those involved in the transport of phosphate. Trivalent methylated arsenicals are also readily transported across cell membranes. However, pentavalent oxygenated arsenicals are poorly transported across the cell membranes, if at all. In contrast, trivalent and pentavalent thiolated arsenicals are readily transported across cell membranes (Suzuki et al., 2010; Cohen et al., 2013). The trivalent and pentavalent thiolated arsenicals are similarly toxic to cells, unlike the distinction between trivalent and pentavalent oxygenated arsenicals (Suzuki et al., 2010; Cohen et al., 2013). The thiolated arsenicals may be toxic to cells because they are readily taken up by cells and quickly converted to the corresponding trivalent oxygenated form of arsenic, which would produce the high toxicity (Suzuki et al., 2010).

It has been repeatedly demonstrated in a variety of systems that trivalent arsenicals readily react with free thiol groups, whether in small molecules such as glutathione, cysteine, or acetylcysteine, or free thiol groups in proteins or small peptides (Cohen et al., 2013; Kitchin and Wallace, 2005). It appears that the biologic effects of arsenic are a consequence of the reaction with these thiol groups (Fig. 1). The difference in reactivity between different cell types and different animal species is related to the availability of free thiol groups, particularly vicinal thiol groups, that are available in the proteins in the respective target tissues. There is considerable variability for specific proteins not only between cells, but even for the same protein in different species. For example, the alpha chain in rat hemoglobin has an extra free thiol group in contrast to other species, such as humans. As a consequence, arsenic is tightly bound to the hemoglobin of rat, acting as a sequestering site for the arsenic (Lu et al., 2007). Binding to other types of proteins also varies between species. Needless to say, these differences would lead to marked differences in toxicity between species.

Additional organic forms of arsenic have been identified in various species and food sources, most of which have little or no toxicity (Cohen et al., 2013; Thomas, 2014). Arsenobetaine is commonly present in seafood, particularly shellfish, but has little or no toxicity. Similarly, arsenosugars have been identified in numerous sources, especially sources of food, but apparently have little or no toxicity. Thus, evaluating arsenic exposure in epidemiology studies requires a careful delineation of speciation of arsenic rather than an assessment of total arsenic either in urine, blood, or toenails, common samples used to ascertain exposure levels for humans. In drinking water, arsenic is present nearly entirely as inorganic arsenic. This is not the case for exposures in foods, which can confound epidemiology studies considerably, especially at low exposures to arsenic in water, when arsenic in food becomes the predominant exposure (Aylward et al., 2014).

3. Arsenic carcinogenesis: cytotoxicity and regeneration

The mode of action for cancer induced by arsenicals appears to involve cytotoxicity with consequent regeneration (Cohen et al., 2006, 2013). This has been most extensively investigated in the urinary bladder (Fig. 2), but similar findings have been identified for the lung and skin (Dodmane et al., 2013; Cohen et al., 2013). A difficulty in studying the mode of action of arsenic carcinogenesis has been the limitations of animal models for the induction of cancer (IARC, 2012). The clearest model of carcinogenesis by any arsenical is that of DMA^V orally administered to rats in the diet or drinking water (Cohen et al., 2006). In rats, it produces tumors of the urinary bladder, but this does not occur in mice. The mode of action for DMA for the rat urinary bladder involves reduction to DMA^{III}, excretion and concentration in the urine, reaction with critical protein sulfhydryl groups in the target tissue, the urothelium, with consequent cytotoxicity, regenerative proliferation and ultimately the formation of tumors. Inhibition of the levels of DMA^{III} in the urine by co-administration with 2,3-dimercaptopropane-1-sulfonic acid (DMPS) leads to complete inhibition of the cytotoxicity and regenerative proliferation following DMA^V administration (Cohen et al., 2002). The lowest effect level is 10 mg/L of the diet, below which the urinary concentration of DMA^{III} is less than 0.1 μmol/L, there is no cytotoxicity and there is no increased proliferation (Cohen et al., 2002, 2006). In contrast, administration of MMA^V does not lead to an increase in tumors of any tissues in rats or mice (Arnold et al., 2003).

The cytotoxicity produced in the bladder following administration of inorganic arsenic to rats or mice in either the drinking water or diet has a no observed adverse effect level (NOAEL) of 2 ppm of arsenic (2000 parts per billion) (Suzuki et al., 2010; Yokohira et al., 2011). This is similar to the no effect level of DMA^V in the rat (Cohen et al., 2006). In contrast to DMA^V, however, inorganic arsenic produces similar effects in the rat and in the mouse (Cohen et al., 2013). The difference between DMA^V and inorganic arsenic effects on the mouse are likely due to differences in metabolism and toxicokinetics.

In vitro systems have been utilized to assess the potency of the toxicity of the various arsenicals. As indicated above, pentavalent oxygen-containing organic arsenicals are weakly cytotoxic requiring concentrations of approximately millimolar to produce cytotoxicity (Cohen et al., 2013). Such levels are not attainable *in vivo*. In contrast, the trivalent arsenicals, whether arsenite, MMA^{III}, or DMA^{III}, are comparably cytotoxic to urothelial cells, whether rat or human (Cohen et al., 2013; Dodmane et al., 2013). Likewise, similar concentrations produce cytotoxicity in human keratinocytes from the skin and human bronchial epithelial cells (Dodmane et al., 2013). The response of these different epithelia is similar to arsenite, MMA^{III}, and DMA^{III}, with MMA^{III} and DMA^{III} usually slightly more toxic than inorganic arsenite. Thus, it can be concluded that trivalent arsenicals are the forms of arsenic that produces the toxicity, and the effects are similar in urothelium, skin, and lung epithelia.

Although these observations regarding cytotoxicity have been established *in vitro*, there is strong evidence that similar processes occur *in vivo* in tissues at high exposures of inorganic arsenic (Cohen et al., 2013; Arnold et al., 2013). The mode of action for arsenic-induced cancer *in vivo* appears to involve cytotoxicity and regenerative proliferation (Figs. 1 and 2). This is also true in humans (Cohen et al., 2013). For example, in the lung, there is increasing evidence that chronic bronchitis and bronchiectasis are associated with increasing exposures to inorganic arsenic (Mazumder et al., 2000; Mazumder, 2007; Milton and Rahman, 2002; Parvez et al., 2008, 2010; von Ehrenstein et al., 2005). These disorders involve toxicity, inflammation and regenerative proliferation. In the skin, the changes of arseniasis have been described for many decades and involve hyperkeratosis with hyper- or hypopigmentation (Tseng et al., 1968; Schuhmacher-Wolz et al., 2009; Melkonian et al., 2011). Associated with the hyperkeratosis, which is a response to keratinocyte toxicity, is a chronic inflammatory response along with hyperplasia of the epidermis, the type of reaction characteristic of cytotoxicity and regeneration. For all of these tissues, prolonged exposure and continued proliferation appears to be required for cancer to develop.

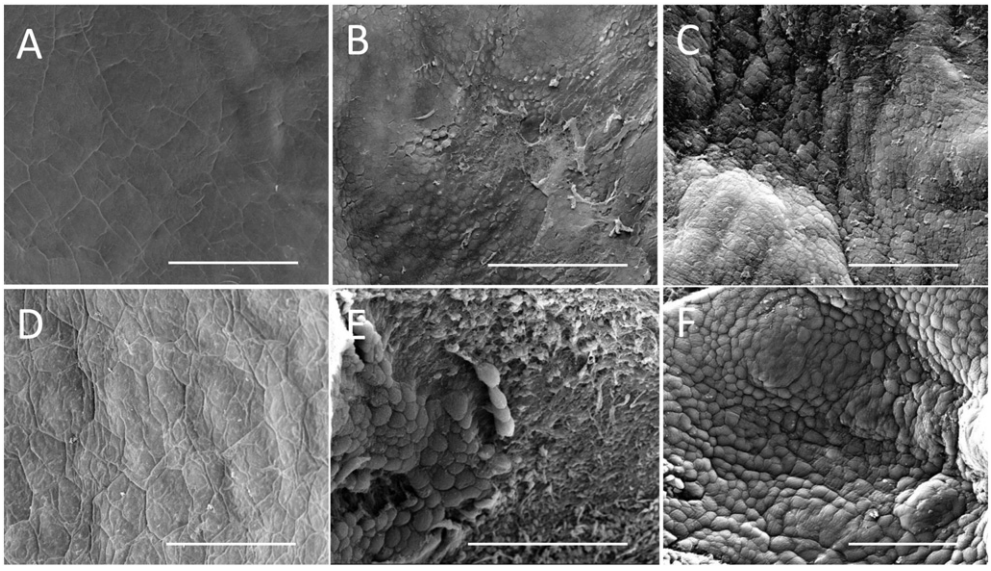

Fig. 2 – Scanning electron micrographs from rats (A–C) and mice (D–F) treated as controls (A and D) or with 100 mg/L arsenic (as arsenite) in the drinking water showing superficial cytotoxicity (necrosis), (B and E) and regenerative proliferation (piling up of cells) (C and F). Bar = A, 200 μm; B, 400 μm; C, 200 μm; D, 200 μm; E, 300; F, 500 μm.

4. Epidemiology

The evidence is strong and accumulating that arsenic-induced carcinogenesis of urinary bladder, skin and lung involves cytotoxicity and regeneration, which would fit a non-linear, threshold dose response (Cohen et al., 2013; Dodmane et al., 2013; Yager et al., 2013). The *in vitro* concentration of trivalent arsenicals required for an adverse effect appears to be 0.1 μmol/L or higher (Gentry et al., 2010). This appears to be true for a wide variety of cell types. Concentrations less than 0.1 μmol/L are adaptive, not adverse, and concentrations ≥10 μmol/L are lethal (although lethality may occur at lower concentrations depending on cell type and arsenical that is present). To attain such levels in human tissues, such as blood or urine, appears to conservatively require human exposure levels of approximately 100 to 150 μg/L in the drinking water (Cohen et al., 2013). It might be higher for certain tissues, such as the lung. This fits with the epidemiology that has been demonstrated for arsenic in a variety of studies (Cohen et al., 2013; Tsuji et al., 2014a). As mentioned above, the studies showing an association between cancer and inorganic arsenic exposure have involved exposures in the drinking water usually of 300 μg/L or higher (Chen et al., 1985; NRC, 2001; Mink et al., 2008; Cohen et al., 2013; Tsuji et al., 2014a), but occasionally down to levels of 100 to 200 μg/L (Lamm et al., 2014, 2015). Studies involving exposures at lower levels, less than 100–200 μg/L, do not show an increase in cancer risk, whether in the bladder (Mink et al., 2008; Tsuji et al., 2014a), lung (Lamm et al., 2014, 2015), or skin (Byrd et al., 1996; Cohen et al., 2013; Lamm et al., 2014). For the skin, which is the most readily observable target tissue, this also corresponds to studies indicating that approximately 100 μg/L in drinking water or higher are required for the development of arseniasis skin changes which represents the precursor change to carcinoma (Haque et al., 2003).

The relationship of arsenicals to cancer has been known for more than a century. It was first clearly shown in patients treated with Salvarsan (arsephenamine), the arsenical developed by Ehrlich for the treatment of syphilis (Cullen, 2008). The number of cases was few and quantitative exposure was not determined (Neubauer, 1947). The first quantitative assessment of arsenic and cancer was based on ingestion of Fowler's solution (Fierz, 1965; Byrd et al., 1996). The association of cancer to arsenic in mining occupations was suggested as early as the 1880s, but it was eventually recognized to be predominantly in smelters (Neubauer, 1947).

Meta-analyses have also supported a threshold effect between 100 and 150 μg/L. For example, a study by Mink et al. (2008) showed no increased cancer risk at concentrations below 100 μg/L. Even stronger evidence was provided by Tsuji et al. (2014a) in a recent meta-analysis demonstrating that the dose response for arsenic in drinking water and urinary bladder cancer is incompatible with a linear, no threshold dose response. Analysis by Lamm et al. (2014), providing a more detailed evaluation of the southwest Taiwanese population, shows a threshold of approximately 150–200 μg/L. In the studies by Lamm and colleagues, there actually appears to be a decreased risk at lower exposures.

5. Arsenic and genotoxicity

The association of inorganic arsenic carcinogenesis with genotoxicity has been extensively evaluated, also. Based on the anionic nature of arsenicals, it is highly unlikely that there would be a direct interaction between arsenicals and DNA. This was confirmed in studies by Nesnow et al. (2002) demonstrating that DNA reactivity does not occur with arsenicals. A direct interaction of arsenicals with DNA would lead to formation of DNA adducts, and no arsenic containing DNA adduct has been reported.

Indirect forms of genotoxicity have also been evaluated, including extensive evaluation of the possible role of oxidative stress. *In vitro*, there is no question that oxidative stress can be shown to be involved with some of the toxicity changes, and these changes can be prevented by co-administration in the culture medium with antioxidants (Basu et al., 2001; Cohen et al., 2013). In contrast, studies *in vivo* have shown little or no role of oxidative damage in arsenic-induced toxicity or carcinogenesis (Suzuki et al., 2009; Cohen et al., 2013). Co-administration with a variety of antioxidants has not led to an appreciable decrease in effects *in vivo* in a variety of modal systems. In addition, recent epidemiology studies in the Bangladesh population (Harper et al., 2014) and in Canada (Normandin et al., 2014) show no relationship between markers of oxidative stress and the development of cancer secondary to exposures of arsenicals. As recently described in *Nature*, there appears to be a myth that has gained strength over the last several decades relating oxidative stress and cancer (Scudellari, 2015), whereas, studies investigating the role of various antioxidants in humans have not found an effect. In many instances there has been a deleterious effect by exposure to various antioxidants (Scudellari, 2015).

The reaction of trivalent arsenicals with sulfhydryl groups could potentially lead to an indirect form of genotoxicity (Cohen et al., 2013). For example, it has been well known for several years that trivalent arsenicals react with tubulin, which is a major component of the mitotic spindle and of cilia. This reaction with cilia might actually be part of the process for induction of toxicity with the bronchial epithelium which is ciliated. A reaction of arsenicals with tubulin could lead to mitotic arrest. Rather than being a cause of genotoxicity, this more likely could be a cause of cytotoxicity. It is unlikely that it would actually lead to an induction of cancer, since other mitotic spindle inhibitors, such as colchicine, have been used for decades in the treatment of gout with no known carcinogenic effect.

Likewise, interaction with a variety of DNA repair proteins could potentially lead to an increased risk of genotoxicity (Banerjee et al., 2008; Cohen et al., 2013). Although there has been a demonstration of reaction with specific DNA repair enzymes, an effect on DNA repair in whole tissue or *in vivo* situations has yet to be demonstrated. This is likely due to the extensive DNA repair network available, including considerable redundancy.

In vitro, genotoxicity has been extensively investigated utilizing assays for mutagenesis, micronucleus formation, or chromosomal aberrations (Cohen et al., 2013). Studies regarding direct mutagenicity, such as the Ames assay involving Salmonella strains or *Escherichia coli* (*E. coli*), have generally been negative, which would be anticipated given the lack of reactivity of arsenicals with DNA.

In contrast, numerous *in vitro* assays have shown positive results when evaluating micronuclei or chromosomal aberrations (Mahata et al., 2004). However, in the *in vitro* studies, concentrations are usually extremely high, frequently greater than 100 μmol/L, but always higher than 10 μmol/L, which would be lethal (Kligerman et al., 2003; Gentry et al., 2010). In fact, for the trivalent arsenicals, cytotoxicity and cell death would likely occur in these studies at much lower concentrations, but cell death may not be readily evident in the short exposure time of many of these assays. Thus, it is difficult to conclude that there is genotoxicity based on *in vitro* assays involving evaluation with micronuclei or chromosomal aberrations.

In vivo, there have also been investigations examining micronucleus formation, and less commonly, chromosomal aberrations given the limitations of such assays in non-hematopoietic cells (Basu et al., 2001; Mahata et al., 2004; Mazumder et al., 2013). In animal studies, the positive results have been at extremely high doses, doses that would be expected to produce cytotoxicity and cell death. As is well known in genotoxicity assays, using guidelines put forward by Organization for Economic Cooperation and Development, examination of cytotoxicity must be performed since it will lead to false positives. This is true whether cytotoxicity is due to necrosis or apoptosis.

6. Genotoxicity studies in humans

During the past two decades, there have been a number of publications examining micronucleus formation in one of the target tissues in arsenic carcinogenesis, the urothelium (Basu et al., 2001, 2002, 2004; Ghosh et al., 2006, 2007, 2008; Moore et al., 1997; Paul et al., 2013). These studies are based on examination of urothelial cells that have been exfoliated into the urine so that they can be readily collected in urine specimens. Most of these studies have been performed in populations in West Bengal (Basu et al., 2001, 2002, 2004; Ghosh et al., 2006, 2007, 2008; Mahata et al., 2004; Paul et al., 2013), but occasionally in other populations as well, such as in Chile (Moore et al., 1997). However, there are numerous difficulties in the interpretation of these studies. To begin with, they have not shown a dose response in relation to arsenic exposure as assessed by either drinking water levels or measurement of urinary arsenic. For example, Basu et al. (2004) evaluated the relationship of inorganic arsenic to micronuclei in exfoliated urothelial cells, buccal mucosal squamous cells, and in blood lymphocytes. The prevalence of micronuclei per 1000 cells in populations with drinking water levels of 50–150, 151–250, or >250 μg/L, was 6.30, 6.48, and 6.98 in urothelial cells, respectively, 5.75, 5.78, and 5.90 for buccal cells, and 9.01, 9.39, and 9.42 for blood lymphocytes. Standard errors were not presented.

As described above, there are many difficulties in the assessment of arsenic exposures. To begin with, exposure in the drinking water is usually assessed at one time point, and does not take into account the various exposures that might occur in an individual over time. Recent evidence indicates that there is significant variability in urinary arsenic in individuals over time, even day to day (Wang et al., 2016). Most epidemiology investigations utilize an assessment of arsenic exposure only once, whether in drinking water or in urine. This likely produces a significant exposure assessment bias. Examining total arsenic in the urine as a measure of arsenic exposure is quite limited, because of the marked variability of other types of arsenicals that can occur in the diet. This is particularly true if there is a substantial amount of seafood or various plant materials such as rice and certain vegetables, which contain variable and high levels of various

organic arsenicals which contribute to the total arsenic in the urine and are unrelated to exposure to inorganic arsenic (Thomas, 2014; Aylward et al., 2014).

Furthermore, the populations that are examined are in different locations, so that there could be numerous confounding factors. The lack of a dose response suggests that these confounding factors, whatever they might be, are actually the variables that are contributing to the differences in micronuclei assessed in the urothelial cells rather than exposure to inorganic arsenic. Exposure to tobacco products is one possibility, since tobacco is well known to affect micronucleus formation (Reali et al., 1987; IARC, 2004; Burgaz et al., 1995; Stich and Rosin, 1984). This is particularly likely as a confounding factor in the populations in West Bengal given the extensive use of betel quid in these populations and the relatively high smoking rates (IARC, 2004). The studies that have been reported have not verified tobacco exposure or lack thereof by examination of cotinine or other markers of nicotine exposure. Another possible variable between populations could be folate levels, which are well known to vary between populations and affects arsenic metabolism and toxicity (Gamble et al., 2006, 2007; Peters et al., 2015). Folate is also associated with variability in micronucleus formation (Bull et al., 2012; Lindberg et al., 2007; Kazimirova et al., 2006). In addition, the specimens that are examined contain urothelial cells that have been exfoliated into the urine. Autolysis occurs virtually immediately in cells once they are exfoliated, so that a variety of effects could be occurring that are not reflective of the intact tissue (Cohen et al., 2007).

Assessment of micronucleus number can also be problematic. Most of the studies examining micronucleus formation in urothelial cells and other human cell types in populations exposed to inorganic arsenic have utilized the Giemsa stain. This is a non-specific stain which can stain not only nuclear (DNA) material, but a variety of other cellular components. A recent finding in mice has shown that high levels of exposure to inorganic arsenic produces an accumulation of inorganic arsenic bound to macromolecules which accumulates in inclusions in the cytoplasm (Suzuki et al., 2008; Yokohira et al., 2010, 2011; Dodmane et al., 2014). These inclusions also stain with the Giemsa stain, but do not contain nuclear material as is evident by the negative staining for 4′,6-diamidino-2-phenylindole (DAPI), a more specific stain for DNA, and with its morphology by transmission electron microscopy. Morphologically, the material appears to be lipid in nature, although the specific components of these inclusions have not been identified. However, similar inclusions were observed in urothelial cells of patients treated with high doses of arsenic trioxide for promyelocytic leukemia (PML) (Wedel et al., 2013). Again, these inclusions were positive by Giemsa stain but not by DAPI stain and did not correspond to micronuclei by electron microscopy. By light microscopy these intracytoplasmic inclusions were morphologically similar in appearance to the micronuclei in various publications (Basu et al., 2001, 2002; Paul et al., 2013). Difficulties of utilizing the Giemsa stain for assessment of micronuclei in various tissue have been described by others (Nersesyan et al., 2006, 2014; Soeteman-Hernández et al., 2015). The high variability in the proportion of Giemsa-positive material that are actually micronuclei adds to concerns with the interpretation of the studies in these human populations. The concerns of the differences in population, assessment of arsenic exposure and the evaluation of micronuclei raise considerable doubt as to the reported findings of an association of micronuclei in these urothelial cells with arsenic exposure. Given the lack of a dose response, this is particularly problematic. Additional studies are required to evaluate the purported relationship of arsenic exposure and micronuclei.

7. Conclusions

The overwhelming evidence regarding the mode of action for induction of cancer by inorganic arsenic involves cytotoxicity and regenerative proliferation rather than genotoxicity (Cohen et al., 2013). Given that the biologic responses to inorganic arsenic exposure are due to reaction with sulfhydryl groups, such a mode of action is highly plausible. The lack of reactivity of inorganic arsenic with DNA strongly suggests that for environmental dose–response assessment the mode of action (Fig. 1) for all of arsenic-induced toxicity is similar, whether cancer or non-cancer (Cohen et al., 2013). It would involve reaction of the trivalent arsenicals with thiol groups in critical proteins in various cell types producing a biologic response. If it is in an epithelial system, the biological response is cytotoxicity, cell death, and regenerative proliferation leading to the induction of cancer (Cohen et al., 2013). In other cell types, such as endothelial cells, the response is likely to result in other forms of toxicity (Dodmane et al., 2015), such as atherosclerosis rather than cancer (Sidhu et al., 2015). Nevertheless, the underlying mode of action is the same for all of these toxic responses. For the cancer endpoint, it is actually not cancer that is induced by the arsenic but a variety of toxicities, such as arseniasis skin changes, bronchitis, or urothelial toxicity, which lead to regenerative proliferation and ultimately an indirect induction of cancer. In non-epithelial tissues, the biologic response is some other type of toxicity which does not lead to cancer. Cancer and non-cancer effects involve an initial interaction of trivalent arsenicals with sulfhydryl groups in critical proteins in the target cell population. All of these changes involve a threshold, and evidence indicates that a threshold in humans for biologic effects appears to be 100–200 μg/L in the drinking water, whether for cancer (Tsuji et al., 2014a; Lamm et al., 2007, 2014, 2015; Byrd et al., 1996) or for non-cancer toxicities (Sidhu et al., 2015; Tsuji et al., 2014b, 2015).

The evidence strongly supports a mode of action that has a nonlinear dose response involving a threshold. Epidemiologic studies also support this conclusion.

Statement of interest

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