



Effect of trivalent arsenicals on cell proliferation in mouse and human microvascular endothelial cells



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ABSTRACT

Chronic exposure to high levels of inorganic arsenic (iAs) has been associated with cancerous and non-cancerous health effects, including cardiovascular effects. However, the mechanism for a presumed toxic effect of arsenic on vascular tissue is not clear. Our working hypothesis is that inorganic trivalent arsenic and its methylated metabolites react with cysteine-containing cellular proteins and alter their function leading to adverse events such as cytotoxicity or proliferation. In this study, human microvascular endothelial cells (HMEC1) and mouse microvascular endothelial cells (MFP-MVEC) were exposed to arsenite (iAs^{III}), monomethylarsonous acid (MMA^{III}), or dimethylarsinous acid (DMA^{III}) for 72 h to evaluate cytotoxicity, and for 24, 48 or 72 h to evaluate cell proliferation. Both cell lines showed similar LC₅₀ values, from 0.1 to 2.4 μ M, for all three trivalent arsenicals. The endothelial cells treated with 1 nM to 1 μ M concentrations of the three trivalent arsenicals did not show increased cell proliferation at 24, 48 or 72 h or increased rate of proliferation at 72 h of exposure. Overall, cytotoxicity of trivalent arsenicals to microvascular endothelial cells is similar to their cytotoxicity to epithelial cells, and that these compounds are not mitogenic.

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1. Introduction

As a natural component of the earth's crust, inorganic arsenic (iAs) is present in soil, water and food. Levels of inorganic arsenic in drinking water vary, and in some parts of the world the levels are as high as 900 ppb or higher [10]. Chronic exposure to high levels of inorganic arsenic has resulted in an increased risk of cancerous and non-cancerous effects [10]. Whether health effects can result from chronic exposure to low levels of iAs (<100 ppb) remains a matter of scientific debate [10,32].

Cardiovascular effects due to exposure to high levels of iAs, generally ≥ 100 ppb, are amongst the major investigated non-cancerous effects [10,23,28]. The incidence of cardiovascular

disease (CVD) risk factors, such as diabetes and hypertension, are also suspected to be increased with exposure to high levels of iAs. Increased prevalence of markers of atherosclerosis, such as carotid plaques and increased thickness of carotid intima have been associated with exposure to high levels of iAs [23,24]. Risk of developing microvascular changes, such as renal disease, neurological diseases, and retinopathy were also reported to be greater with high iAs arsenic exposures in drinking water, and the concurrent presence of diabetes augmented these changes [7]. Most of the data are from regions of high iAs water contamination. There is little evidence with regard to any of the CVD-related effects at exposures to low levels of iAs (<100 ppb in drinking water) [23,28,32].

Animal studies have produced conflicting results [5,6,28]. In FVB female mice, chronic exposure to 100 ppb iAs^{III} in drinking water for 22 weeks was shown to induce hypertension and cardiac hypertrophy [25]. Chronic exposure to 200 or 1000 ppb iAs in drinking water also showed development of atherosclerotic plaques in the macrovasculature of ApoE^{-/-} mice [20]. Mice administered 20 mg/kg of sodium arsenite in water showed increased vascular leakage [5]. At a similar dose, iAs had anti-angiogenic activity in solid tumor models by decreasing expression of vascular endothelial growth factor (VEGF) [34].

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Soucy et al. [26] reported that vessel growth in fibroblast growth factor-2 (FGF-2)-containing Matrigel implants was increased in mice exposed to 5–500 ppb iAs in drinking water for 5 or 20 weeks, but the response decreased with time. However, iAs^{III} alone did not induce neovascularization; rather, it enhanced the effect of FGF-2 in the Matrigel. In addition, the concentration of FGF-2 in the Matrigel was significantly higher than normally occurs in mice or in humans.

The pathogenesis of atherosclerosis has traditionally fallen under two overarching hypotheses: one theory stresses intimal cellular proliferation while the other premise emphasizes recurring thrombi organized over time into clinically significant plaques [28]. Various cellular mechanisms for arsenic-induced effects on the microvasculature have been suggested [2,3,22,29], including increased vascular oxidative stress, increased vascular inflammatory response, induction of endoplasmic reticulum stress, activation of G-protein coupled receptor activation, and stimulation of vascular redox signaling [29]. Pro- and anti-angiogenic responses have been reported when mouse aortic endothelial cells were exposed to high doses of iAs *in vitro* [22,29]. Stimulation of cell proliferation by low concentrations of iAs has been observed in endothelial cells, specifically, in porcine aortic endothelial cells *in vitro* [2], human microvascular endothelial cells (HMEC1) [13], and human lung microvascular endothelial cells [17], possibly secondary to oxidative stress. However, observations of oxidative stress *in vitro* secondary to exposure to arsenicals are not always expressed in a similar manner *in vivo* [10]. Furthermore, a recent study showed that in Bangladesh, in a high arsenic exposed population, an association with markers of oxidative stress was not found [14].

Liver sinusoidal defenestration was reported in a mouse model treated with 10–250 ppb iAs in drinking water [30]. However, the response in the mouse liver appeared to be transient, and furthermore, none of the hepatic sequelae, such as steatosis, fibrosis or cirrhosis, that usually are associated with these vascular changes were observed in longer term studies in mice [31,33].

The applicability to humans of these models in which the effects of iAs on the endothelium were evaluated, is questionable. Mouse models of cardiovascular abnormalities or other inflammatory changes in general do not appear to be representative of the disorders in humans [27,28] so that extrapolation of the results to human risk assessment must be made with caution.

Mammals metabolize inorganic arsenic to mono-, di- and trimethylated compounds of pentavalent arsenic, with mono- and dimethylated compounds of trivalent arsenic formed as intermediates. Numerous studies have shown that the trivalent forms, iAs^{III}, MMA^{III}, and DMA^{III} are reactive and cytotoxic to several epithelial cell types, in contrast to the corresponding pentavalent forms that are not toxic [9]. Further, studies have shown that trivalent methylated forms (MMA^{III}, DMA^{III}) are somewhat more cytotoxic than iAs^{III} to human keratinocytes, urothelial cells, and bronchial epithelial cells, the target tissues for iAs-induced cancer [11]. A similar toxicity pattern has been demonstrated for rat aortic macrovascular endothelial cells [15].

In the present studies, we examined the proliferative response of mouse and human microvascular endothelial cells to the trivalent arsenicals (iAs^{III}, MMA^{III} and DMA^{III}) as well as the cytotoxicity (LC₅₀) of the trivalent arsenicals in the same cells.

2. Materials and methods

2.1. Chemicals and reagents

Sodium arsenite (NaAsO₂, purity 99%) was purchased from Sigma (St. Louis, MO). MMA^{III} and DMA^{III} were synthesized by Dr. William Cullen (University of British Columbia, Vancouver,

Canada), and supplied as the di-iodide and mono-iodide, respectively. The identity and purity of the chemicals were determined by NMR analysis at the University of British Columbia and accepted without further testing. MMA^{III} and DMA^{III} were stored desiccated in the dark at approximately 4 °C.

2.2. Cell culture

The human microvascular endothelial cell (HMEC1) line was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). HMEC1s were isolated from human foreskin dermal tissue and immortalized by the transfection of the transforming protein, SV40 large T antigen [1]. The HMEC1 line was grown in RPMI medium (Hyclone, Thermo Fisher Scientific, Rochester, NY) containing fetal bovine serum (5% v/v) (Atlanta Biologicals, Atlanta, GA), supplemented with 5 ml L-glutamine (MediaTech, Herndon, VA), 100 units/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Grand Island, NY) at 37 ± 1 °C in an atmosphere of 95% air and 5% CO₂.

The mouse mammary fat pad microvascular endothelial cell line (MFP MVEC) was created from cells isolated from the mammary fat pad (MFP) tissue of two female H-2Kb-ts-A58 mice (Immortomice, Charles River Laboratories, Wilmington, MA) [19]. Briefly, Immortomice carry a temperature-sensitive mutant of SV40 large T antigen under the control of the H-2Kb promoter (activated by interferon-γ), which can be used to conditionally immortalize isolated primary cells when grown at the permissive temperature of 33 °C [16]. MFP MVECs were cultured on 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) coated flasks in DMEM (Hyclone Laboratories, Logan, UT) supplemented with 2 mM GlutaMaxTM, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 1x MEM vitamins, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (FungizoneTM), 40 µg/mL gentamicin (all from Invitrogen, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals) in an atmosphere of 95% air and 5% CO₂ at 33 °C for routine expansion and at 37 °C for at least 5 days before experiments.

2.3. Determination of cytotoxicity (LC₅₀)

The number of cells required to be seeded on a 96-well plate to reach approximately 80% confluence at the end of 96 h of plating with a change of medium at 24 h after seeding was first determined. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to varying concentrations (HMEC1, 0.1–50 µM; MFP MVEC, 0.005–50 µM) of trivalent arsenicals at 24 h after plating and continued for 72 h. The cell viability after 72 h treatment was normalized to control. The data were analyzed by non-linear regression analysis (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA). The cytotoxic response of the trivalent arsenicals was compared based on the resulting LC₅₀ values.

2.4. Determination of cell proliferation by flow cytometry

The cells were plated in 6-well plates. To maximize the detection sensitivity, the cells were cultured in a low FBS-containing medium (1% FBS for HMEC1, 2.5% for MFP-MVEC). First, the number of cells required to be seeded in low FBS-containing medium that would reach 80% confluence at the end of 72 h was determined. The cells were seeded and immediately exposed to different concentrations of trivalent arsenicals (HMEC1, 1–1000 nM; MFP-MVEC, 1–500 nM). At 72 h after treatment, the cells were labeled with bromodeoxyuridine (BrdU) for 1 h to incorporate into DNA of cells. Cells were then harvested for flow cytometry analysis as

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