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Expression of selected pathway-marker genes in human urothelial cells exposed chronically to a non-cytotoxic concentration of monomethylarsonous acid

Matthew Medeiros[a,*], Tam Minh Le[a], Daniel Troup[a], Petr Novak[b], A. Jay Gandolfi[a]

[a] Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721, United States
[b] Biology Centre ASCR, Institute of Plant Molecular Biology, Ceske Budejovice, 37005, Czech Republic

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ABSTRACT

Bladder cancer has been associated with chronic arsenic exposure. Monomethylarsonous acid [MMA(III)] is a metabolite of inorganic arsenic and has been shown to transform an immortalized urothelial cell line (UROtsa) at concentrations 20-fold less than arsenite. MMA(III) was used as a model arsenical to examine the mechanisms of arsenical-induced transformation of urothelium. A previous microarray analysis revealed only minor changes in gene expression at 1 and 2 months of chronic exposure to MMA(III), contrasting with substantial changes observed at 3 months of exposure. To address the lack of information between 2 and 3 months of exposure (the critical period of transformation), the expression of select pathway marker genes was measured by PCR array analysis on a weekly basis. Cell proliferation rate, anchorage-independent growth, and tumorigenicity in SCID mice were also assessed to determine the early, persistent phenotypic changes and their association with the changes in expression of these selected marker genes. A very similar pattern of alterations in these genes was observed when compared to the microarray results, and suggested that early perturbations in cell signaling cascades, immunological pathways, cytokine expression, and MAPK pathway are particularly important in driving malignant transformation. These results showed a strong association between the acquired phenotypic changes that occurred as early as 1–2 months of chronic MMA(III) exposure, and the observed gene expression pattern that is indicative of the earliest stages in carcinogenesis.

1. Introduction

Arsenic is a ubiquitous environmental metalloid. Chronic exposure to low concentrations of inorganic arsenic increases risks for the development of skin, lung, and bladder cancers [1,2]. The exact biological events by which inorganic arsenic causes cancer have not been fully elucidated. However, oxidative stress, increased cell proliferation, inhibited DNA repair, genotoxicity, and altered cellular signals have all been suggested as critical events in arsenic-induced carcinogenesis [3–5].

Most human exposure to arsenic is via ingestion of inorganic arsenate [As(V)] and arsenite [As(III)]. Biotransformation of these inorganic arsenic species in humans

* Corresponding author at: Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, United States. Tel.: +1 520 626 7317.
E-mail addresses: Medeiros@pharmacy.arizona.edu, medeiros412@cox.net (M. Medeiros).

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produces methylated metabolites, that are found in the urine of arsenic-exposed individuals. As the urine accumulates in the bladder, the bladder urothelium is clearly exposed to the inorganic arsenic species as well as the methylated metabolites [6–8]. Additionally, *in vitro* studies of urothelial cells (UROtsa) demonstrated that these cells accumulated a greater amount of arsenic species when compared to human primary hepatocytes [9].

Both the trivalent arsenic metabolites, monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)], have been shown to be more cytotoxic than both the methylated pentavalent forms and inorganic arsenic [10–12]. In particular, MMA(III) has been shown to be a potent generator of reactive oxygen species (ROS), and appears to be more clastogenic than inorganic arsenic [13–15]. Cytotoxicity assays of inorganic arsenic and MMA(III) have been performed in UROtsa, where MMA(III) had a 20-fold more toxic response than As(III), supporting earlier work that methylated trivalent arsenic species were highly toxic to immortalized rat (MYP3) and human (1T1) urothelial cells [16,17]. The IC_{50} value for MMA(III) was determined to be 5 μM in UROtsa cells, with no significant effects observed on cell morphology and mitochondrial activity during 24 h of exposure [16]. Thus 0.05 μM (50 nM) has been used in this current study and several others [18–21] as a non-cytotoxic concentration for long-term exposure studies.

Inorganic arsenic and its methylated metabolites have been shown to modify the expression of genes, and cause distinct profiles of cellular responses in urothelial cells and keratinocytes [22–25]. MMA(III) appeared to exhibit a greater carcinogenic potential, more pro-inflammatory signals, and altered expression of growth factors in human keratinocytes compared to inorganic arsenic exposure. Likewise, fundamental similarities and differences were found between As(III)-, MMA(III)-, and DMA(III)-induced transcriptional perturbations in urothelial cells (SV-HUC-1), and that some of these changes were attributed to epigenetic alterations [24]. Even with these bodies of work, the understanding of the relationship between chronic MMA(III) exposure and the development of bladder cancer is still limited.

UROtsa cells chronically exposed to 50 nM MMA(III) for a period of 3 months underwent phenotypic changes consistent with malignant transformation, i.e., increased rate of proliferation, anchorage-independent growth, and formation of tumors in immuno-compromised mice [21]. These acquired phenotypic changes were sustained even after withdrawal of MMA(III). Additionally, chronic exposure to MMA(III) had substantially impacted the global expression of genes at 3 months of exposure with roughly 8.4% of genes significantly changed from 30,000 genes assessed [26]. Surprisingly, significantly altered genes in the first and second months of exposure amounted to less than 1% of the total genes assessed. The transition from a normal to a malignant phenotype occurred between the second and third months of exposure. The early changes described by the gene array analysis implicated the involvement of perturbed immune and inflammatory pathways and related signaling. Similar observations of altered pro-inflammatory genes have been observed previously using the same model [18,19].

Since the gene array study in UROtsa cells was only performed at monthly intervals, resolving the window of transformation between 2 and 3 months of exposure into shorter intervals should define the specific changes that induce malignant transformation with chronic MMA(III) exposure. Therefore, the goal of the present study was to dissect the gene expression changes at weekly intervals in UROtsa cells chronically exposed to a non-cytotoxic concentration of MMA(III) between 2 and 4 months of exposure to resolve the changes contributing to malignant transformation. The study used a customized PCR array to profile the expression of selected genes associated with several important pathways, i.e., apoptosis, immune/inflammatory response, and proliferation as wells as correlate the phenotypic changes that occur within that timeframe. Concomitantly, cell growth kinetics, anchorage-independent growth, and tumorigenicity in SCID mice were also assessed to determine the earliest and persistent phenotypic changes and their association with the changes in expression of selected genes.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution (containing penicillin, streptomycin, and amphotericin B), and 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) were acquired from Gibco Invitrogen/Molecular Probes Corporation (Carlsbad, CA). Diiodomethylarsine [MMA(III) iodide, CH_3AsI_2] was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the technique of Millar et al. [27]. Dissolution of diiodomethylarsine in water yields monomethylarsonous acid [MMA(III)] and the concentration of MMA(III) was verified using high performance liquid chromatography-inductively coupled mass spectrometry (HPLC ICP-MS) in the analytical core of our NIEHS Superfund Research Program. MMA(III) solutions in distilled, de-ionized water were stable for approximately 4 months at 4 °C with no degradation observed when monitored using HPLC ICP-MS [28].

2.2. Preparation of MMA(III) dosing solution

Preparation of dosing solution and procedures were derived from Bredfeldt et al. [16]. Pure MMA(III) was stored in ampules at 4 °C. Fresh stock solutions of 25 mM MMA(III) were made and diluted to a final concentration of 5 μM prior to dosing (1:100 dilution) to obtain a final concentration of 50 nM MMA(III) in the culture media. The level of MMA(III) used in this study (50 nM) represents a relevant physiological concentration as this specific metabolite has been detected in the urine of humans with MMA(III) levels at or near 50 nM when exposed to environmentally relevant concentrations of inorganic arsenic in their drinking water [29,30]. All dosing solutions were sterile filtered with a 0.2 μM Acrodisc (Sigma–Aldrich) and stored in sealed,