Cardiac Epithelial-Mesenchymal Transition Is Blocked by Monomethylarsonous Acid (III)

Tianfang Huang*,1, Joey V. Barnett†, and Todd D. Camenisch*,‡,§,¶,∥

*Department of Pharmacology and Toxicology College of Pharmacy, University of Arizona, Tucson, Arizona 85721, †Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232, ‡Southwest Environmental Health Sciences Center, University of Arizona, Tucson, Arizona 85721, §Steele Children’s Research Center, University of Arizona, Tucson, Arizona 85724, ¶Sarver Heart Center and ∥Bio5 Institute, University of Arizona, Tucson, Arizona 85721

1To whom correspondence should be addressed. E-mail: camenisch@pharmacy.arizona.edu.

ABSTRACT

Arsenic exposure during embryonic development can cause ischemic heart pathologies later in adulthood which may originate from impairment in proper blood vessel formation. The arsenic-associated detrimental effects are mediated by arsenite (iAsIII) and its most toxic metabolite, monomethylarsonous acid [MMA (III)]. The impact of MMA (III) on coronary artery development has not yet been studied. The key cellular process that regulates coronary vessel development is the epithelial-mesenchymal transition (EMT). During cardiac EMT, activated epicardial progenitor cells transform to mesenchymal cells to form the cellular components of coronary vessels. Smad2/3 mediated TGFβ/H2 signaling, the key regulator of cardiac EMT, is disrupted by arsenite exposure. In this study, we compared the cardiac toxicity of MMA (III) with arsenite. Epicardial progenitor cells are 15 times more sensitive to MMA (III) cytotoxicity when compared with arsenite. MMA (III) caused a significant blockage in epicardial cellular transformation and invasion at doses 10 times lower than arsenite. Key EMT genes including TGFβ ligands, TβRIII, Has2, CD44, Snail1, TBX18, and MMP2 were down regulated by MMA (III) exposure. MMA (III) disrupted Smad2/3 activation at a dose 20 times lower than arsenite. Both arsenite and MMA (III) significantly inhibited Erk1/2 and Erk5 phosphorylation. Nuclear translocation of Smad2/3 and Erk5 was also blocked by arsenical exposure. However, p38 activation, as well as smooth muscle differentiation, was refractory to the inhibition by the arsenicals. Collectively, these findings revealed that MMA (III) is a selective disruptor of cardiac EMT and as such may predispose to arsenic-associated cardiovascular disorders.

Key words: arsenicals; MMA (III); TGFβ; cardiovascular development

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Arsenic is a naturally occurring toxic metalloid that is released from geological deposits and enters the ground water. Drinking water and food are the major sources of human exposure to environmental arsenic (Jiang et al., 2012). Established as a class 1 human carcinogen, inorganic arsenic has been reported to cause cancers in skin, lung, bladder, and liver (Smith et al., 1992). Low-to-moderate levels of chronic arsenic exposure are also associated with non-cancerous ailments, especially in the cardiovascular system (States et al., 2009). Emerging data suggest that adult diseases have developmental origins, and early life environmental exposures affect not only embryonic development but also initiate and pattern the molecular programming of diseases in adulthood. The disruption in normal developmental physiology eventually leads to chronic conditions such as metabolic disease, hypertension, and cardiovascular disease later in life (Farzan et al., 2013). A case study in Chile reported two children died from myocardial infarction and vascular lesions caused by early life arsenic poisoning (Rosenberg, 1973). Another study also reported that in utero exposure to arsenic induced early onset of atherosclerosis in ApoE-/- mice (Srivastava et al., 2009). Together, these reports suggest a strong association between arsenic and developmental origins of cardiovascular disorders. A major contributor to ischemic heart pathologies and cardiovascular mortality is coronary artery disease; however, the
influences due to environmental arsenic in this disease process are unknown. Similarly, the impact of toxicants on blood vessel formation and function during development has not been well characterized.

After absorption into the body, arsenate (As(V)) is first reduced to arsenite (As(III)), then methylated by arsenic(+3)-methyltransferase (AS3MT) to form metabolites that include monomethylarsonic acid (MMA) and dimethylarsinous acid (DMA), which are excreted in the urine (Meza et al., 2004). Although methylation of inorganic arsenic facilitates excretion, and it is considered to be a detoxification mechanism, highly reactive intermediate MMA (III) has been reported to induce severe cytotoxicity in hepatocytes (Petrick et al., 2000). Elevated MMA (III) levels in the urine and tissues also lead to a higher risk of arsenic-associated skin lesions (Yu et al., 2000). During pregnancy, arsenic and its metabolites can easily pass through the placenta to the fetus. Compared with the later stages of life, the fetus is exposed to relatively higher level of arsenic and MMA (III) during early gestation as a result of the insufficient methylation of transferred inorganic arsenic (Vahter, 2009). However, the potential adverse effects caused by MMA (III) on fetal growth and especially on the development of the cardiovascular system have not been studied.

Coronary artery development begins with a portion of cardiac progenitor cells derived from the epicardium undergoing an epithelial-mesenchymal transition (EMT). These cells transform into fibroblast-like mesenchyme, migrate and invade the myocardium, and differentiate into several cardiac cell types including smooth muscle cells and endothelial cells, which comprise cellular components of coronary vessels (Austin et al., 2008). We previously demonstrated that the TGFβ ligands, especially TGFβ2, are key inducers of developmental cardiac EMT in mice (Camienisch et al., 2002). The canonical TGFβ pathway is mediated by phosphorylated Smad2/3 effectors which translocate to the nucleus and regulate the expression of genes that determine cell differentiation and extracellular matrix remodeling (Allison et al., 2013; Kurisaki et al., 2001). Smads are not the only downstream effectors of TGFβ2; Smad-independent non-canonical pathways such as the mitogen-activated protein kinase (MAPK) pathway also participate in TGFβ signaling. Smad3 activation is required but not sufficient to induce TGFβ-mediated EMT, indicating a co-regulation of non-canonical effectors (Itosh et al., 2003; Yu et al., 2002). The MAPKs and Erk5 are also required for the disassembly of cell adherens junctions and induction of cell motility mediated by TGFβ, as its activation regulates a subset of target genes that regulate cell-matrix interactions, cell motility, and endocytosis (Gui et al., 2012). We have previously reported that low level arsenic exposure disrupted canonical TGFβ2 signaling via reduced Smad2/3 phosphorylation and nuclear translocation (Allison et al., 2013). Here we investigate the impact of MMA (III) on TGFβ2 mediated EMT during early events critical for coronary vessel development. Our observations suggest that low level arsenical exposure selectively disrupts non-canonical MAPK in addition to blocking canonical Smad2/3 activation during cardiac EMT. However, differentiation into smooth muscle is refractory to the effects of MMA (III) exposure.

**MATERIALS AND METHODS**

**Cell line and cell culture.** Conditionally immortalized epicardial cells were harvested and cultured as previously described (Austin et al., 2008). Briefly, epicardial progenitor cells were generated from transgenic mice that carried a temperature sensitive promoter driven SV40 large T antigen. For experiments, epicardial cells were moved to 37°C condition, where the thermolabile promoter of SV40 antigen was silenced reverting cells to a primary cell state. Complete cell culture Dulbecco’s modified Eagle’s medium media containing 10% fetal bovine serum, antibiotics, insulin–transferrin–selenium (Invitrogen, Carlsbad, CA), and mouse gamma interferon (10 U/ml, R&D systems, Minneapolis, MN) was used to maintain cells.

MTS cell viability assay. Cells at 80% confluence were serum starved for 6 h before exposure to indicated doses of arsenite or MMA (III) for 24 or 48 h. Cell viability was assessed based on relative mitochondrial activity which was measured by the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega G5421). The absorbance of the bioreduced MTS was detected at 490 nm in an M2 Spretramax plate reader (Molecular Devices). Cell viability analysis and calculation of IC50 values were described previously (Allison et al., 2013). All assays were performed in triplicate at each concentration of arsenite or MMA (III) in a minimum of three independent determinations. Statistical significance was determined by two-tailed Student’s t-test.

**Real-time PCR.** 80% confluent cells were serum starved for 6 h before exposure to arsenite or MMA (III) for 24 h. Total RNA was extracted using the RNA-STAT60 reagent following the manufacturer’s instructions (Tel-test, Invitrogen). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time PCR was performed using TaqMan Master primer-probe system (Roche). The housekeeping gene 40S Ribosomal Protein 7 (RPS7) was used as an internal calibrator control for gene expression analysis. Heme oxygenase 1 (Hmox1) was used as a positive control as it responds to arsenite induced oxidative stress. The following TaqMan primers were designed to detect the indicated genes:

Western blot analysis. Serum starved cells were exposed to arsenite or MMA (III) for 24 h followed by TGFβ2 stimulation for the indicated time periods. Whole cell protein lysates were prepared in RIPA buffer containing 10mM sodium phosphate (pH 8.0), 150mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) (Lau et al., 2010). Protease inhibitor and phosphatase inhibitors (phenylmethylsulfonyl fluoride, sodium fluoride (NaF), and vanadate at 1μM final concentration) were added into RIPA buffer. To detect nuclear expression of selected proteins, nuclear fractionation of protein lysates was performed as previously described (Haspel and Darnell, 1999). Cell lysates or nuclear fractions were resolved by 10% SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane. The membranes were blocked with 5% BSA/TBST and then incubated with different antibodies diluted in 3% BSA/TBST (1:000 dilution for all primary antibodies and 1:3000 dilution for all secondary antibodies). Immunoblotting detection was performed using chemiluminescence reagent (100mM Tris pH 8.5, 250mM luminol, 92mM p-coumaric acid, and 0.018% H2O2) as previously described (Sollome et al., 2014). Images were detected with ChemiDoc XRS + (BIO-RAD) and image quantification was analyzed by Image Lab Software. Antibodies against p-Smad2/3 (sc-11769), Smad2/3 (sc-133098), p38 (sc-535), p-Erk5 (sc-135760), Erk1/2 (sc-292838), LaminA (sc-20680), GAPDH (sc-365062), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology. Antibodies detecting p-p38 (no. 9211),...
Collagen gel invasion assay. In order to determine the detrimental effects of arsenicals on epicardial cell invasion, fluorescently labeled cells were placed onto collagen gels casted in the upper chamber of a transwell 96-well plate. Cells were then given 1.34 μM arsenite or 0.134 μM MMA (III) with 2 ng/ml TGFβ2 for 48 h. The extent of cellular invasion was quantified by detecting fluorescent signals in the lower chamber of the transwell system as previously reported (Craig et al., 2009). Detection of specific fluorescence was determined at 538 nm using a Spectramax Gemini plate reader (Molecular Devices, Sunnyvale, CA).

Immunostaining. An equal number of epicardial cells were seeded on Rat Tail Type I Collagen gels (BD Biosciences, Franklin Lakes, NJ) within a glass cylinder to set up culture for the EMT-collagen gel assay. After epicardial cells formed a monolayer with a defined cell edge on the gel surface, they were subjected to the indicated conditions. Cells on collagen gels were fixed in 4% PFA at room temperature for 15 min. Fixed cells were then permeabilized with 0.5% Triton X-100/PBS for 10 min followed by rinsing with 1× PBS for 5 min. Cells were blocked in 5% BSA/PBS for 2 h at room temperature and incubated with β-catenin primary antibody (sc-31001, Santa Cruz Biotechnology) in 1:100 dilution at 4°C overnight. Cells were subjected to six washes in 1× PBS, and primary antibody was detected with a 1:500 dilution of goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells were then subjected to six washes followed by nuclei staining, using 4,6-diamidino-2-phenylindole (DAPI, Sigma), for 30 min at room temperature. Collagen gels were mounted on glass slides and the morphology of epicardial cells was detected by β-catenin staining and distribution. Fluorescence microscopy was performed using a Leica DMLB microscope and documented using a Retiga 200R camera and Image-Pro Plus 5.1 software.

SM22α reporter assay. Epicardial cells isolated from SM22α-LacZ reporter transgenic mice were generated and isolated as described (Compton et al., 2007). This cell line has been established as a cellular model to determine epicardial cell differentiation into a smooth muscle cell lineage in vitro. SM22α-LacZ epicardial cells were pre-exposed with arsenite or MMA (III) for 18 h and subsequently stimulated with 2 ng/ml TGFβ2 for 24 h to allow transformation. X-GAL staining was performed using the β-galactosidase reporter gene staining kit (Sigma-Aldrich) to detect SM22α promoter activity. Relative expression of SM22α reporter gene was detected by real-time PCR by standard methods and primers illustrated in Table 1.

RESULT

MMA (III) Exhibits Higher Cytotoxicity on Epicardial Cells Compared with Arsenite

Monomethylarsonous acid [MMA (III)] is a toxic arsenic species that is generated during arsenite metabolism. The toxicity of MMA (III) on epicardial progenitor cells is not known. We investigated epicardial cell viability exposed to arsenite or MMA (III) at 24 and 48 h. The ranges of concentrations studied were from 0 to 20 μM for sodium arsenite and 0 to 1.2 μM for MMA (III). Figure 1 shows arsenite impairing cell viability starting at 3 μM after exposure with an IC50 value of 11.8 μM at 24 h and an IC50 of 7.8 μM at 48 h. This is consistent with a previous report on arsenic toxicity on epicardial cells (Allison et al., 2013). We detect higher sensitivity to MMA (III) exposure by epicardial cells compared with the parent compound arsenite. We found the IC50 value for MMA (III) cytotoxicity at 24 h to be 0.6 μM, and the IC50 at 48 h to be 0.5 μM. These results show that epicardial cells are approximately 15 times more sensitive to MMA (III) induced cytotoxicity compared with arsenite.

Arsenicals Block the Expression of Key Genes that Regulate Developmental EMT

We have previously reported that arsenite disrupts some of the key genes that regulate EMT in a dose-dependent manner (Allison et al., 2013); however, the role of MMA (III) in regulating EMT gene expression is not known. We examined a representative panel of EMT-related genes. The TGFβ family of molecules, including the TGFβ ligands TGFβ1 and TGFβ2, and the key ligand presenting receptor TβRII, are known to function in cell differentiation and invasion during organogenesis (Compton et al., 2007). Has2, which is the major enzyme that synthesizes hyaluronan, and its receptor CD44 have been previously reported to stimulate epicardial cell invasion (Craig et al., 2010). The TGFβ effector Snail1 is an essential transcription factor that induces EMT in both developmental and cancer models (Medici et al., 2008; Tran et al., 2011). The T-Box factor, TBX18, and Matrix Metalloproteinases, MMP2, also function in developmental
TABLE 1 Primers for EMT Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>RPS7</td>
<td>5′-AGCACGTGGTCTTCATTGCT-3′</td>
<td>5′-CTGTCAGGGTACGGCTTCTG-3′</td>
</tr>
<tr>
<td>Has2</td>
<td>5′-GGCGGAGGACGAGTCTATG-3′</td>
<td>5′-ACACAAGAACAATCTCAAGTGC-3′</td>
</tr>
<tr>
<td>CD44</td>
<td>5′-TCCCTTCTTATCCGGAGCAC-3′</td>
<td>5′-ACGTCTCCTGCTGGGTAGC-3′</td>
</tr>
<tr>
<td>Snail1</td>
<td>5′-ACCTGCTCGGTCTCAGTC-3′</td>
<td>5′-ATTGCAGTGAGGGCAAGAGA-3′</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>5′-TGGAGCAACATGTGGAACTC-3′</td>
<td>5′-GTCAGCAGCCGTTACCA-3′</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>5′-TGGAGTTCCAGACACTCAACACA-3′</td>
<td>5′-AAGCTTCGGGATTTATGGTGT-3′</td>
</tr>
<tr>
<td>TβRIII</td>
<td>5′-TCCAAACATGAAGTCCA-3′</td>
<td>5′-GTCCAGGCCGTGGAAAAT-3′</td>
</tr>
<tr>
<td>TβRI</td>
<td>5′-GCAAGCTCCTCATCGTGTTG-3′</td>
<td>5′-AGAGGTGGCAGAAACACTGTAAT-3′</td>
</tr>
<tr>
<td>TβRII</td>
<td>5′-AGAAGCCGCATGAAGTCTG-3′</td>
<td>5′-GGCAAACCGTCTCCAGAGTA-3′</td>
</tr>
<tr>
<td>MMP2</td>
<td>5′-TAAACCTGGATGCCGTCGT-3′</td>
<td>5′-TTCAGGTAATAAGCACCCTTGAA-3′</td>
</tr>
<tr>
<td>TBX18</td>
<td>5′-CAGACTCTCGGGAGAAGACS-3′</td>
<td>5′-TATGGGCTCAGACCTTGA-3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-CTCGGCTCGGGAAAATTTTG-3′</td>
<td>5′-CTCGTCTCTCACCCTTGA-3′</td>
</tr>
<tr>
<td>Hmox1</td>
<td>5′-GTCAAGACAGGCTGACAGA-3′</td>
<td>5′-ATCACCTGCAGCTCCTCAAA-3′</td>
</tr>
<tr>
<td>SM22α</td>
<td>5′-CCTTCCAGTCCAACGCGAC-3′</td>
<td>5′-GTAGGATGGACCTTTGTTG-3′</td>
</tr>
</tbody>
</table>

EMT (Duong and Erickson, 2004; Keichi et al., 2013). Vascular endothelial growth factor (VEGF) has been identified as an inhibitor of developmental EMT as previously reported by our laboratory (Dor et al., 2003). Also, because increased expression of Hmox1 is associated with the response to arsenic exposure, Hmox1 was used as a positive control in the gene expression analysis (Allison et al., 2013). The effects of MMA (III) exposure on the expression of the EMT programmed genes were observed 24 h after exposure, and 1.34μM arsenite was used as a control for comparison. These concentrations were used because they are below the IC50 concentrations, respectively. As shown in Figure 2, both arsenicals significantly inhibit the expression of TGFβ ligands, TβRIII, Has2, CD44, as well as Snail1, TBX18, and MMP2. MMA (III) exhibited a similar inhibitory effect in a much lower dose compared with arsenite exposure. In contrast, we detect an induction in VEGF expression consistent with its role in down-regulating EMT. Collectively, we detect a dramatic reduction in the pro-EMT gene program following exposure to MMA (III) with a concurrent substantial increase in VEGF, a negative regulator of EMT.

MMA (III) Inhibits TGFβ2-Induced N-Cadherin Expression and Cellular Invasion

N-cadherin is a cell adhesion molecule known to increase with TGFβ stimulation. However, the expression of N-cadherin in response to TGFβ2 stimulation during epicardial EMT and the regulatory effect of arsenicals on it have not been elucidated. To address this question, we treated epicardial cells with 2 ng/ml TGFβ2 in the presence or absence of arsenite or MMA (III) for 48 h. As shown in Figure 3A, TGFβ2 induces a dramatic increase in N-cadherin detection (Fig. 3A, lane 2) compared with control. In contrast, MMA (III) is substantially reduced following arsenite and MMA (III) exposure (Fig. 3A, lanes 3 and 4). MMA (III) decreased N-cadherin expression more profoundly than arsenite. Also, MMA (III) and arsenite decrease N-cadherin basal protein level compared with untreated group (Fig. 3A, lanes 5 and 6), indicating that arsenicals were able to block endogenous
Fig. 2. Arsenicals inhibit the expression of genes that regulate EMT. Epicardial cells were treated with 1.34 μM NaAsO₂ or 0.13 μM MMA (III) for 24 h. RPS7 was used as an internal control for normalizing all target mRNA expression levels. Relative mRNA expression of EMT programmed genes was compared to untreated samples through quantitative real time PCR. Hmox1 was used as a positive control for responsiveness to arsenite. Samples were evaluated in triplicate with a minimum of three independent experiments. * p value <0.05.
FIG. 3. MMA (III) blocks mesenchymal cell differentiation and cell invasion in epicardial cells. Epicardial cells were untreated or stimulated with 2 ng/ml TGFβ2, TGFβ2 combined with either 1.34 μM NaAsO2 or 0.134 μM MMA (III), NaAsO2, or MMA (III) alone for 48 h. Immunoblot detects N-Cadherin and β-Actin for loading control (A). Epicardial cells labeled with calcinAM fluorescent dye were seeded in 96-well transwell plate and subject to the indicated conditions for 48 h. Relative fold change in cell invasion compared to control was detected (experiments were repeated 3 times in triplicate, #=p<0.001, indicates TGFβ2 stimulates significant increase in cell invasion compared to UTX group. *p<0.005, indicates significant blockage in cell invasion with arsenicals co-exposure compared to TGFβ2 stimulation.) (B). Collagen gel spot culture assays were performed to visualize the morphology of transformed epicardial cells through the detection and expression pattern of β-catenin. Cells were exposed to 1.34 μM NaAsO2 or 0.134 μM MMA (III) in the presence or absence of TGFβ2 for 48 h. Immuno-detection of β-catenin (shown in red) highlights the cobblestone sheet of undifferentiated epicardial cells compared to differentiated cells in panel 4 (white arrows). Nuclei were stained with DAPI (C). Blind cell counting was performed in each condition. Bar graph shows the fold change in transformed mesenchymal cell numbers relative to control group. (#=p<0.00063, indicates TGFβ2 stimulates significant increase in mesenchymes formation compared to UTX group. *p<0.0065, indicates significant disruption in mesenchymal cells transformation with arsenicals co-exposure compared to TGFβ2 stimulation (D).
growth factors induced N-cadherin expression without affecting epicardial cellular viability. To determine if N-cadherin expression correlates with less mesenchymal cell production, invasive cell motility was detected in the presence or absence of MMA (III) (Fig. 3B). TGFβ2 stimulates cellular invasion by 40% in the modified Boyden chamber assay, whereas arsenite and MMA (III) exposure during TGFβ2 stimulation ablates this invasive cell motility. Arsenite or MMA (III) alone had no effect on the basal level of cell motility. In order to visualize cell morphology of transforming epicardial cell under each condition, spot culture assays were performed as previously described (Allison et al., 2013). Epicardial cell monolayers with a defined cell boundary were exposed to arsenicals in the presence or absence of TGFβ2. As shown in Figure 3C, epicardial cells were activated and transformed into fibroblast like mesenchymal cells following TGFβ2 stimulation. In contrast, arsenical exposure profoundly blocked cell transformation as less transformed cells were observed. The change in mesenchymal cell numbers between groups was quantified as shown in Figure 3D. TGFβ2 induced a 10-fold increase in mesenchymal cell production compared with control which was almost completely blocked by arsenical exposure. Thus, MMA (III) is a highly potent arsenical species that silenced developmental cardiac EMT.

Smooth Muscle Differentiation is Refractory to MMA (III) Toxicity

We previously detected that arsenite exposure does not affect TGFβ2-induced smooth muscle differentiation in epicardial cells (Allison et al., 2013). Because epicardial cells do not metabolize arsenite, experiments were performed to determine the impact of MMA (III) on smooth muscle differentiation. Smooth muscle actin 22α is a marker for differentiated vascular smooth muscle cells (Compton et al., 2006). SM22α-LacZ epicardial cells derived from transgenic mice were exposed to 1.34μM arsenite or 0.134μM MMA (III) for 18 h followed by TGFβ2 stimulation for 24 h. β-Galactosidase staining was used to detect the expression of LacZ. As shown in Figure 4A, TGFβ2 induces a dramatic expression of β-galactosidase which indicates activation of SM22α promoter and the smooth muscle cell phenotype. This induction is not abrogated by MMA (III) exposure. To further assess SM22α promoter activity, real-time PCR analysis for SM22α reporter mRNA was performed. As shown in Figure 4B, TGFβ2 induced SM22α gene mRNA and this expression was not blocked by arsenicals exposure indicating that smooth muscle differentiation is refractory to the effects of arsenicals.

Arsenate and MMA (III) Block Smad2/3, Erk1/2, and Erk5 Phosphorylation, But not p38 Phosphorylation

The activation of the TGFβ pathway leads to phosphorylation of downstream canonical and non-canonical effectors. We have previously reported that arsenite blocks the Smad2/3-dependent TGFβ2 canonical pathway (Allison et al., 2013). Erk1/2, Erk5, and p38 MAPKs are involved in TGFβ2-induced EMT in tumor models (de la Cruz-Merino et al., 2009; Dreesen and Brivanlou, 2007). In order to investigate whether these non-canonical MAPK cascades respond to TGFβ2 stimulation compared with canonical Smad2/3 in epicardial cells, a time course following TGFβ2 stimulation was performed to detect phosphorylation of Erk5, Erk1/2, and p38. As shown in Figure 5A, Smad2/3 and Erk5 respond to TGFβ2 stimulation in a similar manner. Detection of phosphorylated Smad2/3, and Erk5 starts at 10 min after TGFβ2 stimulation, and the phosphorylation level persists with the presence of TGFβ2. Duration of Erk1/2 activation is fast and short which only occurs within the first 5 min upon TGFβ2 stimulation. In contrast, p38 responds to TGFβ2 in a biphasic manner. A quick induction is detected at 1 min after TGFβ2 stimulation, this phosphorylation disappears and is detected again at 30 min and persists out to 1 h. The detection of the phosphorylation pattern of Erk1/2, Erk5, and p38 following TGFβ2 stimulation allows for the determination of the effects of arsenicals on their activation. Epicardial cells were pre-exposed to 1.34μM arsenite, 0.07μM, or 0.13μM MMA (III) for 24 h. Each condition was then stimulated with TGFβ2 and compared with untreated controls. As shown in Figure 5B, a 20-fold lower concentration of MMA (III) blocked the phosphorylation of Smad2/3. Both arsenite and MMA (III) decreased TGFβ2-induced activation of Erk1/2 and Erk5. However, the activation of p38 appears to be refractory to this inhibition by MMA (III) and arsenite.

Arsenicals Selectively Block Smad2/3 and Erk5 Nuclear Translocation

To determine whether the blockage in activation of Smad2/3 and Erk5 leads to decreased nuclear localization, we performed parallel experiments as described in Figure 5, and prepared lysates from nuclear fractions for the detection of Smad2/3, Erk5, and p38. As shown in Figure 6, TGFβ2 induced an increase in Smad2/3 and Erk5 nuclear localization (Fig. 6, lane 2); however, arsenicals dramatically block this event (Fig. 6, lanes 3, 4, and 5). MMA (III) exhibited the same inhibitory effect as arsenite but at a much lower concentration, and in a dose-dependent manner (Fig. 6, lanes 4 and 5). Both arsenite and MMA (III) showed no effect on p38 nuclear translocation.

DISCUSSION

Chronic arsenic exposure through contaminated water has been linked to ailments in adults (Abernathy et al., 1999; Naujokas et al., 2013). Incidence of spontaneous abortion, low birth weight, and fetal malformations associated with arsenic exposure during pregnancy suggests that arsenic also has developmental toxicity (Aggarwal et al., 2007; Farzan et al., 2013). Methylation of inorganic arsenic has been considered a major metabolic mechanism to convert arsenic into more readily excretable forms; however, it is not always a detoxification process. Studies have shown that mono-methylated arsenic, especially the trivalent form, MMA (III), elicits higher cytotoxicity and is a more active carcinogen compared with the parent form of arsenic (Petrick et al., 2000; Steinmaus et al., 2006; Yu et al., 2000). MMA (III) induces malignant transformation of urothelial cells (UROtsa), a well-established cell model for studying bladder cancer, at concentrations 20-fold less than arsenite (AsIII) (Medeiros et al., 2012). Similarly, we detect the cytotoxic IC50 value of MMA (III) to be 20 times lower compared with the IC50 value of AsIII (at 24 h). Although an increase in cell cytotoxicity is seen with the longer arsenite exposure at 48 h, MMA (III) elicits higher toxicity by 24 h. This indicates that MMA (III) is a potent and cytotoxic agent (Fig. 1).

Arsenic-related toxicity through MMA (III) has drawn increased interest in an effort to understand its detrimental effects (Ge et al., 2013; Rahman and Hassler, 2014). Due to kidney filtration, the bladder is the primary exposure site for accumulated MMA (III). The mechanism of MMA (III) in triggering malignant urothelial transformation during bladder cancer has been well studied (Medeiros et al., 2012; Wang et al., 2007; Wnek et al., 2010). There is limited data showing how MMA (III) potentiates skin cancer (Chen et al., 2008; Delker et al., 2009), or the role of MMA (III) in other arsenic induced diseases. One report on nanomolar levels of MMA (III) impairing vascular smooth muscle contractility in mice suggests that MMA (III) contributes to arsenic-associated cardiovascular diseases more potently than arsenite.
FIG. 4. Smooth muscle differentiation is not blocked by MMA (III). Transgenic smooth muscle 22α-LacZ epicardial cells were left untreated or pretreated with 1.34 μM NaAsO₂ or 0.13 μM MMA (III) for 18 h followed by co-treatment with 2 ng/ml TGFβ2 for 48 h. X-gal staining was applied on fixed cells to detect the SM22α-LacZ activity (A). mRNA gene expression of smooth muscle 22α (SM22α) was measured and normalized to RPS7, *p<0.05, NS=p>0.05 (B).
FIG. 5. Arsenicals block TGFβ2 induced phosphorylation of Smad2/3 and Erk1/2 and Erk5 but not p38. Epicardial cells were stimulated with 3 ng/ml TGFβ2 for 0-60 mins. Whole cell lysates were subjected to immunoblotting analysis (A). Epicardial cells were either left untreated or pre-treated with 1.34 μM NaAsO₂, 0.067 μM MMA (III), or 0.13 μM MMA (III) for 24 h and subsequently stimulated with 3 ng/ml TGFβ2 (15 min for Smad and Erk5 activation, 2 min for Erk1/2 and p-p38 activation). Whole cell lysates were subjected to immunoblotting with antibodies against phosphorylated and total forms of Smad2/3, Erk1/2, Erk5, and p38 as well as β-actin (B).
Arsenicals selectively block Smad2

**FIG. 6.** Arsenicals selectively block Smad2

Epicardial cells were exposed to the indicated doses of NaAsO₂ or MMA (III) for 24 hours followed with or without 3 ng/mL TGFβ2 stimulation (20 min for Smad2/3 and Erk5 nuclear translocation, 45 min for p38 nuclear translocation). Nuclear fractions were subjected to western blotting with antibodies against Smad2/3, Erk5, p38, and LaminA. Although whole cellular p-Erk5 is modestly reduced, detection of nuclear Erk5 is dramatically decreased in MMA (III) exposed samples (A). Densitometry analysis of Smad2/3/LaminA, Erk5/LaminA, and p38/LaminA were performed. Relative densities of targets bands in 1.34 μM As + TGFβ2, 0.067 μM MMA (III) + TGFβ2, 0.13 μM MMA (III) + TGFβ2 groups were compared to the 3 ng/mL TGFβ2 group. Data were analyzed from 3 independent experiments using a two tail Student’s t-test. *p<0.01, NS=p>0.36. (B)

(Bae et al., 2008). Both inorganic arsenic and MMA pass through the placental membrane, and embryonic progenitor cells are much more sensitive than terminally differentiated adult cells (Allison et al., 2013; Jin et al., 2006). However, the developmental toxicity of MMA (III) has not been extensively investigated. We show here that MMA (III) inhibits coronary progenitor cell EMT, which may lead to altered coronary vasculature during fetal development. Exposure to MMA (III) at substantially lower levels than arsenite causes significant decrease in production of invasive mesenchyme by TGFβ2 stimulation. Canonical Smad activation triggered by TGFβ2 is blocked by MMA (III) exposure. Erk1/2 as well as Erk5 MAP kinase activation, which compromise non-canonical TGFβ2 signaling, are also affected by both arsenite and MMA (III). However, MMA (III) has no impact on cardiac smooth muscle differentiation, or p38 signaling, as both remain intact despite arsenite or MMA (III) exposure. This suggests that arsenicals do not uniformly disrupt signaling cascades associated with developmental EMT.

EMT is a cellular process where epithelial cells lose cell polarity and adhesion to basement membrane to gain migratory and invasive properties of mesenchymal cells which can differentiate into multiple cell types (Austin et al., 2008; Carew et al., 2012; Compton et al., 2006). EMT plays an essential role in embryogenesis, tissue repair and regeneration, fibrosis, and cancer metastasis (Galichon and Hertig, 2011; Janda et al., 2002; Kalluri and Weinberg, 2009). EMT is classified into three subtypes based on the stimuli and context (Carew et al., 2012). Type 1 EMT, also referred as developmental EMT, occurs during early embryogenesis. In heart development, endocardial EMT contributes to endocardial cushion development and valve formation, whereas epicardial EMT contributes to coronary vessel development and valve remodeling (Allison et al., 2013; Azhar et al., 2011; Lencinas et al., 2013). Type II and type III EMT occur during adulthood contributing to tissue fibrosis and cancer (Carew et al., 2012). Arsenicals can induce fibrosis and cancer in multiple cell lines and animal models, indicating a contribution to detrimental type II and type III EMT; however, the role of arsenicals in type I developmental EMT remains poorly understood (Ghatak et al., 2011; Sarin et al., 1999; Wang et al., 2013; Xu et al., 2012b). We investigated MMA (III) effects on a comprehensive set of type I EMT genes (Fig. 2). There is a profound inhibition in the mRNA expression of TGFβ2 and TβRIII with MMA (III) (0.13 μM) exposure when compared with the changes in TGFβ1, TβRI, and TβRII. TβRIII serves a distinct role in amplifying TGFβ2 downstream signaling by inducing high affinity binding between TGFβ2 and TβRI and TβRII (Compton et al., 2007). These results suggest that TGFβ2 is a sensitive target to arsenical toxicity, which is consistent with the previous report that TGFβ2, instead of TGFβ1 and TGFβ3, is the key inducer of developmental EMT during early embryogenesis (Camenisch et al., 2002; Doetschman et al., 2012).

The expression of Snail1, a downstream effector of TGFβ2 signaling, is also decreased with MMA (III) exposure indicating an inhibitory effect on TGFβ2 signaling. This coincides with the significant down-regulation in the protein level of N-cadherin (Fig. 3A), which normally increases during cell differentiation and mesenchymal production (Avdal et al., 2007; Nakajima et al., 2004). Disruption in extracellular matrix signaling also occurs with MMA (III) exposure, shown by a decreased mRNA level of Has2 and MMP2. This coincides with less activated epicardial cells and a decrease in invasive mesenchyme as detected by the invasion assays (Fig. 3B–D). Hmx1 is a transcriptional factor that can be activated by cellular oxidative stress (Xin et al., 2014; Yan et al., 2014). The fact that Hmx1 is poorly induced by MMA (III) when compared with arsenite suggests that although MMA (III) is more
cytotoxic, it may induce less reactive oxygen species mediated cellular stress.

Mesenchymal cells produced from activated epicardial cells can invade the myocardium and give rise to smooth muscle cells and form coronary vessels (Compton et al., 2006; Darland and D’Amore, 2001). Although smooth muscle differentiation occurs after EMT, it does not mean that it is regulated by EMT; or that it requires the same regulation as the EMT process. We showed that arsenite has no inhibitory effects on TGFβ2-induced smooth muscle differentiation in epicardial progenitors (Allison et al., 2013). In this study, MMA (III) also does not block smooth muscle differentiation; however it strongly inhibits EMT (Fig. 4). These observations indicate that there is divergence in the signaling pathways involved in EMT and smooth muscle differentiation processes, and that EMT-regulated pathways are selectively inhibited by arsenicals. Other studies have shown similar observation that activation of RhoA and p160 rho kinase is required to induce expression of smooth muscle markers SM22α and SMαA (Compton et al., 2006; Lu et al., 2001; Mack et al., 2001). However, there are conflicting results on the role of p38 in the differentiation of smooth muscle. Stimulation of p38 activity was shown to be associated with smooth muscle de-differentiation and decreased expression of smooth muscle markers (Hayashi et al., 1999, 2001). However, other studies report that p38 activation increases SmαA expression (Compton et al., 2006). In the present study, we show p38 activation in response to TGFβ2 stimulation. This activation and nuclear translocation is not affected by arsenical exposure (Figs. 5 and 6). Together, we show that cellular events and signaling pathways that are related to smooth muscle differentiation remain intact despite arsenical exposure.

The disruption of TGFβ2 signaling in cardiac EMT is caused by down-regulation of its intracellular effectors, Smad2/3. We have previously reported that low level arsenite exposure significantly blocks phosphorylation and nuclear translocation of Smad2/3 (Allison et al., 2013). Here, we observe the same inhibitory effects with MMA (III) at much lower concentration (0.067μM and 0.13μM) (Fig. 5B). Smad2/3 are key transcriptional factors that mediate TGFβ2 stimulation (Gaarenstroom and Hill, 2014). Activation of Smad3 is detected in TGFβ-mediated fibrosis in heart and kidney (Divakaran et al., 2009; Qin et al., 2011). Smad2 activity is required to modulate self-renewal and differentiation of mouse embryonic stem cells (Xu et al., 2012a). On the other hand, overexpression of Smad3 fails to fully induce TGFβ2 mediated EMT, indicating that other non-canonical Smad pathways are also required for diverse cellular events (Townsend et al., 2011).

MAPK activation is part of the non-canonical signaling triggered by TGFβ (Craig et al., 2008; Zhang, 2009). Stimulated TβRI and TβRII recruit and activate the Shc/Grb2/Sos complex. This complex then activates Ras to consequently phosphorylate MAP2K (Raf) which then activates MAPKs (Erk1/2) (Zhang, 2009). Previous work reported that the MAPKs, MEKK3, contributes to cardiac EMT via activation Erk1/2 and Erk5, in response to TGFβ2 (Craig et al., 2010). Erk5 null embryos have deficits in vessel development and heart looping (Regan et al., 2002), and overexpression of Erk1/2 promotes segregation during development of vasculogenesis (Kim et al., 2013). Both Erk1/2 and Erk5 activity are required to mediate TGFβ2-induced hyaluronic acid production and cell differentiation during cardiac EMT (Craig et al., 2010). Erk5 and p38 kinases also translocate into the nucleus to modulate the activity of other related transcription factors and chromatin remodeling enzymes (Craig et al., 2008). In addition, unlike Erk5, Erk1/2 do not have subcellular preference upon stimulation with TGFβ2 (Supplementary fig. 1A). In this study, phosphorylation of Erk1/2 and Erk5 MAPK is inhibited by arsenite and MMA (III) exposure (Fig. 4B). Furthermore, Erk5 nuclear translocation is also dramatically blocked by arsenite and MMA (III) exposure (Fig. 5). Although no clear evidence shows that nuclear expression of Erk5 is required for its signaling transduction, it is possible that nuclear localization may facilitate Erk5 kinase activity in activating other transcriptional factors to regulate gene expression during cardiac EMT.

Collectively, these data demonstrate that MMA (III) exhibits developmental toxicity on select EMT signaling pathways. Down-regulation of TGFβ2, TβRII, Snai1, MMP2, as well as inhibition of Smad2/3, Erk1/2, and Erk5, suggests that these are sensitive targets to environmental insults during cardiac EMT. Activation of these pathways not only contributes to early embryonic development, but is also required to promote cardiac regeneration after injury during adulthood. In addition, we also show that both activation of p38 and smooth muscle differentiation are refractory to effects of arsenical exposure. In conclusion, disruptions in canonical Smad2/3 and non-canonical Erk1/2 and Erk5's signaling are mechanisms for MMA (III) inhibition of developmental cardiac EMT.

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


Ge, Y., Gong, Z., Olson, J. R., Xu, P., Buck, M. J. and Ren, X. (2013). Inhibition of monomethylarsonic acid (MMA(III))-induced cell malignant transformation through restoring dysregulated histone acetylation. Toxicology 312, 30–35.


Keichi, M., Nimura, K., Mori, M., Nakagami, H. and Kaneda, Y. (2013). The transcription factors Tbx18 and Wt1 control
the epicardial epithelial-mesenchymal transition through biregulatory action of Slug in murine primary epicardial cells. PloS One. 8, e57829.


