Published in final edited form as:

Arch Toxicol. 2014 February ; 88(2): 249–261. doi:10.1007/s00204-013-1141-2.

Methylarsonous acid causes oxidative DNA damage in cells independent of the ability to biomethylate inorganic arsenic

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Abstract

Inorganic arsenic (iAs) and its toxic methylated metabolite, methylarsonous acid (MMA^{III}), both have carcinogenic potential. Prior study shows iAs induced malignant transformation in both arsenic methylation-proficient (liver) and methylation-deficient (prostate) cells, but only methylation-proficient cells show oxidative DNA damage (ODD) during this transformation. To further define if arsenic methylation is necessary for transformation or ODD induction, here we chronically exposed these same liver or prostate cell lines to MMA^{III} (0.25–1.0 μ M) and tested for acquired malignant phenotype. Various metrics of oncogenic transformation were periodically assessed along with ODD during chronic MMA^{III} exposure. Methylation-deficient and methylation-proficient cells both acquired a cancer phenotype with MMA^{III} exposure at about 20 weeks, based on increased matrix metalloproteinase secretion, colony formation and invasion. In contrast, prior work showed iAs-induced transformation took longer in biomethylation-deficient cells (~30 weeks) than in biomethylation-proficient cells (~18 weeks). In the present study, MMA^{III} caused similar peak ODD levels at similar concentrations and at similar exposure times (18–22 weeks) in both cell types. At the approximate peak of ODD production both cell types showed similar alterations in arsenic and oxidative stress adaptation factors (i.e. *ABCC1*, *ABCC2*, *GST- π* , *SOD-1*). Thus, MMA^{III} causes oncogenic transformation associated with ODD in methylation-deficient cells, indicating further methylation is not required to induce ODD. Together, these results show that, MMA^{III} and iAs cause an acquired malignant phenotype in methylation-deficient cells, yet iAs does not induce ODD. This indicates iAs likely has both genotoxic and non-genotoxic mechanisms dictated by the target cell's ability to methylate arsenic.

Keywords

arsenic; cancer; malignant transformation; oxidative DNA damage

INTRODUCTION

Arsenic-contaminated drinking water is a common global health problem for millions of people (IARC 2012). Arsenic is carcinogenic in humans with target tissues including the skin, lung, and urinary bladder and potentially the prostate, liver, and kidney (NTP, 2011; IARC, 2012). Inorganic arsenic (iAs) and its methylated metabolite methylarsonous acid (MMA^{III}) both have carcinogenic potential in rodents (Waalkes et al. 2007; Tokar et al. 2010a, 2012, IARC 2012). Exposure to iAs also induces malignant transformation *in vitro* in skin, lung, liver, prostate, or kidney cells (Zhao et al., 1997; Achanzar et al., 2002; Pi et al., 2008; Tokar et al., 2010b; Li et al., 2011; Stueckle et al., 2012). In addition, studies show

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that MMA^{III} can effectively cause malignant transformation *in vitro* in urinary bladder cell lines (Bredfeldt et al., 2006; Wnek et al., 2010). Given its reactivity and toxicity compared with un methylated arsenicals (Stybło et al., 2000), MMA^{III} is believed by some to possibly be an important carcinogenic species. However, the exact carcinogenic species and mechanisms of arsenic carcinogenesis are not fully defined and likely are multi-factorial (IARC 2012).

Multiple endogenous and exogenous factors can stimulate the generation of reactive oxygen species (ROS) in mammalian cells. Oxidative stress and oxidative DNA damage (ODD) likely results once the build-up of ROS overwhelms cellular chemical defense mechanisms, including cellular antioxidants, enzymatic oxidant systems, and DNA repair mechanisms (Valko et al. 2006; Klaunig et al. 2011; Kryston et al. 2011). This imbalance between cellular antioxidant repair systems and ODD can potentially lead to cancer due to accumulation of genetic mutations that can activate oncogenes and/or inactivate tumor suppressor genes (Valko et al. 2006; Klaunig et al. 2011; Kryston et al. 2011). ROS generated during arsenic exposure or arsenic metabolism is suspected to play a role in arsenic-induced carcinogenesis (Valko et al., 2006; Kitchin and Conolly, 2010), although this has not been directly shown in tumor end-point studies. However, studies have shown exposure to iAs or MMA^{III} will induce ODD as a result of ROS generation (Nesnow et al., 2002; Gomez et al., 2006; Kojima et al., 2009; Wnek et al., 2011). At least in some cells, this has been shown to be related to oncogenic transformation, as a blockade of arsenical-induced ODD effectively blocks acquisition of cancer phenotype (Kojima et al., 2009).

Arsenicals can have various effects on the expression and/or function of DNA damage/repair mechanisms and pathways. For instance, phosphatase and tensin homologue (PTEN) is a tumor suppressor gene that is commonly mutated or deleted in cancers, but plays vital roles in proper DNA repair and DNA damage response pathways (Ming and He, 2012). Chronic exposure to arsenic depletes the expression of PTEN during cancer formation *in vivo* and during malignant transformation *in vitro* (Cui et al. 2004; Tokar et al., 2010a; Sun et al. 2012). Thus, not only can exposure to arsenicals induce ROS-mediated ODD (Nesnow et al., 2002; Gomez et al., 2006; Kojima et al., 2009; Wnek et al., 2011), it can also inactivate various factors involved in DNA repair, thereby perturbing the repair process (Cui et al. 2004; Tokar et al., 2010a; Wnek et al., 2011; Sun et al. 2012). These different roles in DNA damage and DNA repair may actually work in combination to facilitate the arsenic-induced oncogenic process. Indeed, arsenic-transformed skin keratinocytes are predisposed to UV-induced ODD but because of prior adaptation to arsenic, are better able to survive a UV exposure insult that kills normal cells, allowing UV-damaged cells to bypass normal cell population check points even though damaged (Sun et al., 2011).

We have variously shown that chronic exposure to iAs induces malignant transformation in both iAs methylation-proficient (ie, liver; Zhao et al. 1997) and methylation-deficient cells (ie, prostate; Achanzar et al., 2002) cells. However, iAs exposure induces a much more rapid transformation concurrently with ODD in the methylation-proficient cells (Kojima et al., 2009). In methylation-deficient cells iAs will induce malignant transformation (Achanzar et al., 2002) but this occurs in the absence of any evidence of ODD and takes ~60% longer than for methylation-proficient cells (29 weeks vs. 18 weeks; Kojima et al., 2009). This suggests that either a methylated arsenical, such as MMA^{III}, may be required for induction of ODD or that the act of methylation itself may generate radicals. To directly test these possibilities, the current study exposed the same arsenic methylation-proficient liver cells (TRL1215) and methylation-deficient prostate cells (RWPE-1) to MMA^{III} for up to 30 weeks. The cell lines were periodically assessed for ODD and acquisition of cancer cell characteristics during this exposure. In addition, several DNA oxidative stress response factors were measured in both cell types during MMA^{III} exposure. Together with our

previous study (Kojima et al., 2009) the current results show that for ODD-associated transformation a methylated arsenic species is required but further methylation is not necessary. ODD-associated arsenical-induced transformation appears more rapid as well. However, neither DNA damage nor arsenical methylation are required for arsenic-induced malignant transformation to occur in a given cell type.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium arsenite and *p*-iodonitrotetrazolium chloride (INT) were obtained from Sigma-Aldrich (St. Louis, MO). 5,5-dimethyl 1-pyrroline *N*-oxide (DMPO) was obtained from Alexis Biochemicals (San Diego, CA). MMA^{III} was a kind gift from Dr. William R. Cullen, University of British Columbia.

Cells and Cell Culture

RWPE-1 cell line is arsenic methylation-incompetent (Benbrahim-Tallaa et al., 2005) and was cultured in complete keratinocyte serum-free medium (KFSM; Gibco/Invitrogen, Carlsbad, CA) with epidermal growth factor (EGF) and bovine pituitary extract (BPE) supplements and 1% antibiotic-antimycotic (Gibco/Invitrogen). The TRL1215 cell is arsenic methylation-proficient (Zhao et al. 1997; Romach et al. 2000) and was cultured in William E medium (Gibco/Invitrogen) containing 10% fetal bovine serum (Gibco/Invitrogen), 31 µg/mL penicillin, and 50 µg/mL streptomycin. Both cell lines were cultured in a humidified atmosphere at 5% CO₂ and 37°C and chronically exposed to 0.0 (control), 0.25, 0.5, or 1.0 µM methylarsonous acid (MMA^{III}) for up to 30 weeks. Medium was changed twice/week and sub-confluent cells were passaged weekly. The concentrations of MMA^{III} used showed no effects on cell growth.

DNA Isolation and Measurement of ODD

Isolation of DNA and measurement of ODD using the IST method were carried out as described in detail previously (Ramirez et al., 2006, 2007; Kojima et al., 2009). The IST method directly measures the formation of DNA-centered radicals by formation of adducts with DMPO, conversion to stable nitron adducts, and subsequent immunochemical quantification. Measurements represent the mean of nine samples from three independent experiments.

Zymographic Analysis for Secreted MMP Activity

Secreted MMP-2 and MMP-9 as combined activity levels were measured using SDS-PAGE zymography as described (Tokar and Webber, 2005) with one minor change for TRL1215 cells. MMA^{III} in the medium of TRL1215 cells appeared to block activity of the MMPs in the conditioned medium. Trivalent arsenicals can inhibit various cellular enzymes (see Jomova and Valko, 2011). Therefore, to obtain an accurate measurement of MMP activity in TRL1215 cells, MMA^{III} was removed from culture medium for 7 days before collection of conditioned medium. MMPs from RWPE-1 cells were not similarly impacted. The difference may be that one group of MMP enzymes is from human cells (RWPE-1) and one is from rodent (TRL1215). For MMP assessment, 5 µg of protein/well from conditioned media was loaded (n = 3). Gels were electrophoresed at 160 V for 1.5 hrs, incubated in renaturing buffer at room temperature (two separate washes, 30 min each), followed by incubation in developing buffer at room temperature (30 min), and then developing buffer at 37°C for 18–20 hrs. Gels were then soaked in SimplyBlue Safestain (Invitrogen) for approximately 4 hrs. Clear bands, indicating MMP activity, were measured by ImageJ

software (NIH). Three independent samples ($n = 3$) were loaded for each treatment and measurements for controls were set at 100%.

Agar Assay

The ability of MMA^{III}-treated cells to grow in an anchorage-independent manner, a common characteristic of cancer cells, was examined using a soft agar assay as described (Tokar and Webber, 2005). Colonies were allowed to form over 21 days and counted with an automated colony counter. Triplicate dishes were prepared for control and treated cells and counts for control were set at 100%.

Invasion Assay

As another indicator of *in vitro* transformation, cellular invasiveness was examined using a modified Boyden chamber assay as described (Tokar and Webber, 2005). Cells that had invaded into and through the membrane and into the bottom chamber were counted. Untreated cells were used as the control and set as 100% for quantitation of results. Triplicate samples of each group were assessed.

Real Time Reverse Transcription Polymerase Chain Reaction

Total RNA was collected from sub-confluent cells using TRIzol reagent (Invitrogen), purified with Rneasy mini kit columns (Qiagen, Valencia, CA), and transcribed with MuLV reverse transcriptase and oligo-DT primers. Primers were designed using Primer Express 3.0 software (Applied Biosystems). Amplifications were done using the Absolute SYBR Green ROX Mix (Abgene, Rockford, IL). Cycle time (Ct) values were normalized to those of β -actin and glyceraldehyde-3-phosphate dehydrogenase in the same sample. Three independent flasks ($n = 3$) for each treatment were collected and Ct values for control cells were set at 100%.

RESULTS

MMA^{III} Induces Similar Oncogenic Characteristics Regardless of Methylation Capacity

Increased secretion of active matrix metalloproteinases (MMPs) is a common occurrence in cancer cells which aids invasion and metastasis. Activity of secreted MMPs was measured in MMA^{III}-exposed cells. Both cell lines showed time-dependent increases in secreted MMP activity during chronic MMA^{III} exposure (Fig. 1). By 20 weeks or more of exposure, these levels (2.1–4.5 fold of control) were equal to or exceeded those typically seen in cells that produce xenograft tumors when transformed *in vitro* with arsenicals (Achanzar et al., 2002; Pi et al., 2008; Kojima et al., 2009; Tokar et al., 2010c; Huang et al., 2011; Sun et al., 2012). Colony formation in soft agar measures the ability of cells to grow in an anchorage-independent manner, a common characteristic of cancer cells. Liver cells began showing an increase in colony formation starting at 5 weeks of MMA^{III} exposure which generally increased thereafter (Fig. 2A). Starting at 15 weeks of MMA^{III} exposure, the prostate cells began showing a time-dependent increase in colony formation (Fig. 2B). Further methylation of MMA^{III} may help explain the earlier increase in colony formation in TRL1215 cells. This observation requires further study with MMA^{III} with an inhibitor of methylation or further methylation to a metabolite such as dimethylarsinous acid (DMA^{III}). The prostate and liver cells also showed very similar increases in invasive ability following exposure to MMA^{III}. Starting at 15 weeks of exposure, both cell lines started showing significant, time-dependent increases in invasive ability to maximal increases of 4.8-fold (TRL1215) and 4.6-fold (RWPE-1) by 30 weeks of exposure (Fig. 2C, D).

MMA^{III} Exposure and Decreased PTEN

PTEN is a tumor suppressor gene commonly decreased in cancer cells including arsenic-induced tumor cells. PTEN is also known to play a key role in DNA damage and repair. MMA^{III} exposure caused comparable, sustained decreases in *PTEN* transcript levels starting at 10 weeks in methylation-deficient RWPE-1 cells and methylation-proficient TRL1215 cells (Fig. 3).

In total, the increased MMP secretion, colony formation, and invasion as well as the loss of PTEN expression between 20 and 25 weeks were similar to or exceeded values for cells that have been shown to have tumor forming capacity in xenograft studies (Achanzar et al., 2002; Pi et al. 2008; Kojima et al., 2009; Tokar et al., 2010c; Wnek et al. 2010; Huang et al., 2011).

MMA^{III} Induces ODD Independent of Ability to Methylate Arsenic

The methylation-deficient prostate and the methylation-proficient liver cell lines showed a strikingly similar ODD pattern during MMA^{III} exposure at the highest concentration of exposure (1.0 μ M; Fig. 4). Both cell lines showed a delayed (2–4 weeks) pattern of increased ODD and surprisingly comparable maximal increases between 18 weeks (RWPE-1, 416%) and 22 weeks (TRL1215, 466%) at the highest (1 μ M) concentration of MMA^{III} used. Both cell lines then showed a precipitous decrease in ODD back to control levels by 30 weeks of exposure (Fig. 4). This pattern of ODD induction occurred in a concentration-dependent manner in both cells lines (Fig. 4).

Direct comparison of concentration-response for ODD at the peak of activity (at around 20 weeks from data in Fig. 4) for the two cell lines proved interesting (Fig. 5). At the highest MMA^{III} concentration used (1 μ M), the peak ODD levels were very similar between the two cell lines. The ODD levels at the two lower concentrations (0.25 and 0.5 μ M) tended to be somewhat lower in the methylating liver cells (30–33% less) than in the non-methylating prostate cells, perhaps indicating that further methylation may produce a metabolite less prone to cause ODD than MMA^{III} and this capacity is not saturated at lower concentrations.

In comparing the present results with MMA^{III} in methylation-proficient and methylation-deficient cells to earlier work with iAs transformation and ODD generation in these same cells (Kojima et al. 2009), several important conclusions become apparent. Firstly, liver (TRL1215) cells that can methylate iAs were transformed at a similar time-point with either iAs or MMA^{III} treatment but showed much more MMP secretion and peak levels of ODD at the approximate point of transformation with iAs exposure (Kojima et al. 2009; Fig. 6A, B). Thus, the formation of MMA^{III} from iAs seems to enhance ODD and cellular aggressiveness. In sharp contrast, methylation-deficient prostate (RWPE-1) cells showed no ODD over baseline with iAs treatment and it took ~30 weeks for them to undergo transformation compared to only 20 weeks to transform these cells with MMA^{III} along with marked increases in ODD (Fig. 6C, D). These distinctions likely have mechanistic significance.

Effects of MMA^{III} on arsenic adaptation- and stress-related factors

Various genes that can become altered in an adaptive response to chronic arsenical exposure or other types of stress were examined during chronic MMA^{III} exposure. For genes associated with arsenical efflux, in prostate cells transcript levels of *ABCC1* were initially decreased (10 weeks) but increased to levels above control at 20 and 30 weeks while levels of *ABCC2* and *GST- π* were increased at 20 and 30 weeks (Fig 7). Transcript levels of all these genes in liver cells were increased at 10, 20, and 30 weeks except for *GST- π* which was increased only at 20 and 30 weeks (Fig. 7). For genes associated with response to

oxidant stress, MMA^{III} exposure caused cell line-specific opposing effects on heme oxygenase-1 (*HO-1*) between the cell lines increasing transcript in prostate cells and decreasing it in liver cells (Fig. 8). *SOD-1* transcript levels increased in both cell lines at 20 and 30 weeks, but while *SOD-2* decreased in prostate cells and showed a decrease only at 10 weeks in liver cells (Fig. 8). *NRF2* showed an early decrease at 10 weeks but returned to control levels by 20 weeks in prostate cells (Fig 8) but showed no change in liver cells during MMA^{III} exposure (Fig. 8).

DISCUSSION

Arsenic is one of the earth's most wide-spread natural environmental toxicants and a known human carcinogen (NTP, 2011; IARC, 2012), making exposure to this metalloid a considerable worldwide health problem. The exact carcinogenic arsenic species and mechanisms of oncogenesis are not fully defined. Based on our previous study using only iAs exposure *in vitro* and arsenic methylation-proficient and -deficient cell lines (Kojima et al., 2009), we hypothesized that a methylated metabolite like MMA^{III} exposure could be able to induce ODD and cause acquisition of a cancer phenotype independent of the arsenic biomethylation ability of the cells. Other work has linked MMA^{III} to cellular DNA damage (Mass et al. 2001; Schwerdtle et al. 2003) *in vitro* but has not linked this even to acquisition of cancer phenotype. To test this hypothesis, the current study chronically exposed the arsenic methylation-deficient prostate (RWPE-1) cells and the methylation-proficient liver (TRL1215) cells to MMA^{III} for up to 30 weeks and examined ODD induction and acquired cancer phenotype. We found that both methylation-proficient and methylation-deficient cells acquired cancer cell characteristics concurrently with ODD during chronic MMA^{III} exposure. These findings, combined with prior work (Kojima et al. 2009), lead to several important conclusions. One is that arsenic methylation is not required for arsenical-induced ODD, as it occurs with MMA^{III} in methylation-deficient cells without further methylation. Secondly, MMA^{III} and iAs can both induce a malignant phenotype in a methylation-deficient, target-relevant cell, but MMA^{III} does so more rapidly and with concurrent ODD (see Fig. 6). This indicates that iAs, with methylation, likely can have both genotoxic and non-genotoxic effects when causing cell transformation, extending and further supporting similar conclusions in earlier work (Kojima et al. 2009). Furthermore, it appears that iAs, with subsequent methylation, greatly enhances ODD, as seen by comparing earlier data from methylation-proficient liver cells exposed to iAs (Kojima et al. 2009) to those from methylation-proficient cells exposed to MMA^{III} exposure in this study (see Fig. 6). This could be important in target cells of arsenic carcinogenesis that do not methylate but may be exposed to methylated compounds generated elsewhere, such as the uroepithelium.

During the multi-step process of carcinogenesis, cells acquire several biological characteristics that allow them to become cancerous by circumventing the constraints of a "normal" phenotype (Hanahan and Weinberg, 2011; Floor et al., 2012). In the current study, both cell lines showed increases in several of these characteristics to levels at or above cells that were malignantly transformed by chronic iAs exposure as established by cellular behavior (invasion, colony formation, MMP secretion, and xenograft formation; Achanzar et al. 2002; Pi et al. 2008; Tokar et al. 2010a; Sun et al. 2012). In this regard, MMPs play many important roles during carcinogenesis and are usually elevated in cancer cells compared to their normal counterparts (Kessenbrock et al. 2010). Invasion into neighboring tissues and metastasis to distant sites are two defining and classical properties of malignant cells (Hanahan and Weinberg, 2011). The ability of cells to grow in an anchorage-independent manner and form colonies in soft agar is commonly used to measure *in vitro* cancer phenotype and is positively correlated with *in vivo* tumorigenicity (Leone et al. 1991). The time-dependent increases seen in these transformation markers strongly suggest that MMA^{III} exposure induced an acquired cancer phenotype in both the arsenic methylating cells

(TRL1215) and the non-methylating cells (RWPE-1). Moreover, the similarities in the increases of cancer cell characteristics between the cell lines suggest that MMA^{III}-induced transformation occurs at a similar time (~20 weeks) and to a generally similar degree of aggressiveness. This is in sharp contrast to a comparison of MMA^{III}-induced transformation and iAs-induced transformation in these same cell lines. The methylation-proficient liver cells showed markedly different increases in the various transformation markers and ODD during exposure to iAs which were much higher than during transformation with MMA^{III} (see Fig. 6; Kojima et al., 2009). MMP secretion and ODD were several fold higher at the approximate point of transformation in the methylation-proficient liver cells treated with iAs compared with MMA^{III} (Kojima et al., 2009). This again indicates the first step in inorganic arsenic methylation as important in the generation of ODD. In contrast, transformation of methylation-deficient RWPE-1 cells not only appeared to take longer with iAs (~30 weeks; Kojima et al. 2009) than with MMA^{III} (current study), but the levels of increased colony formation and MMP secretion were much higher with MMA^{III} transformation than with iAs in these cells (current study; Kojima et al., 2009). So it appears when the non-methylating cells were transformed with an arsenical that causes ODD it clearly does so more rapidly and creates a more aggressive transformant.

PTEN is one of the most commonly mutated genes in human and rodent cancers and plays critical roles in the pathogenesis of this disease (Di Cristofano and Pandolfi, 2000). Further evidence of an acquired cancer phenotype in both cell lines during chronic MMA^{III} exposure is the loss of expression of the *PTEN* tumor suppressor gene. Recent evidence shows that PTEN is also vital for proper DNA damage repair and DNA damage response (Ming and He 2012) and, therefore, its loss or depletion could increase the chance of genomic instability (Kryston et al. 2011). Thus, the loss of this protective factor could help explain the induction of ODD seen in this study. Moreover, this MMA^{III}-induced depletion of PTEN expression is consistent with that seen during malignant transformation and tumor formation by other arsenicals (Cui et al. 2004; Tokar et al., 2010a; Sun et al. 2012), suggesting a common mechanism in several models of arsenical-induced carcinogenesis. Previous studies have shown that arsenicals (iAs, MMA^{III}) can alter signaling via Akt phosphorylation but these exposures had no effect on PTEN levels (Paul et al. 2007). Whether the MMA^{III}-induced PTEN depletion in the current study translates into altered signaling via increased Akt signaling requires further study.

Increased generation of reactive oxygen species (ROS) can overwhelm cellular defense mechanisms and eventually lead to oxidative stress and ODD. ODD plays a critical role in carcinogenesis and frequently results in mutagenesis and transformation (Valko et al. 2006; Klaunig et al. 2011; Kryston et al. 2011). In addition, generation of ODD is often times reliable evidence of oxidative stress in malignantly transformed cells (Kryston et al. 2011). In this regard, ROS-generated oxidative stress and mutation is believed to be to be an important mechanism in arsenic-induced cancers (Valko et al., 2006; Kitchin and Conolly, 2010) as exposure to arsenicals induces ODD in various cells (Nesnow et al., 2002; Gomez et al., 2006; Kojima et al. 2009; Wnek et al., 2011). In the current study, MMA^{III} caused a remarkably similar temporal pattern of ODD generation in both the methylation-deficient cells and methylation-proficient cells. Exposure of these same two cell lines to iAs induced ODD only in the methylation-proficient cell line, although both cell lines could be malignantly transformed by the metalloid (Kojima et al., 2009). Together, these results suggest that a methylated arsenical, such as MMA^{III}, is obligatory for the generation of ODD though it may not be necessary for malignant transformation with iAs. This is an important point because it appears various target cells of arsenic carcinogenesis only poorly methylate iAs, including human prostate epithelial, keratinocytes, bronchial, and bladder epithelial cells (Styblo et al. 2000; Benbrahim-Talla et al. 2005). Additionally, further

methylation to a dimethylated metabolite appears not necessary for malignant transformation, at least in the methylation-deficient cell line used in this study.

Several arsenic adaptation and antioxidant-related factors that likely play a role in arsenic tolerance and perhaps the current observed pattern of ODD generation were altered in both cell lines during MMA^{III}-induced transformation. ABC transporter proteins (ABCC1, ABCC2) and GST- π work together to efflux arsenic and are associated with arsenic tolerance (Kala et al. 2000; Liu et al. 2001; Leslie et al. 2004). These factors and others such as heme oxygenase-1 (HO-1), superoxide dismutases (SOD), and NRF2 play important roles in innate resistance and/or adaptation to arsenic by preventing arsenic-induced oxidative damage and apoptosis (Gottesman et al. 2002; Lee et al. 2003; Tokar et al. 2010c). However, it must be kept in mind that such features allow for generalized resistance to apoptosis, and with this adaptation to arsenic toxicity an expansion of a malignant phenotype can occur (McKenzie and Kyprianou 2006; Tokar et al. 2010c; Hanahan and Weinberg 2011). During MMA^{III}-induced transformation, there were similar expression trends of several adaptation/oxidative stress factors in the cell lines. The lower levels of many of these factors (~10 weeks) may play a role in the induction or early increases of ODD. However, the increases in these factors at 20 and 30 weeks could lower ROS levels which could, in turn, prevent ODD from occurring. The opposing trends of some factors (ie, SOD-2, HO-1) between the cell lines suggest different mechanisms may be at play during MMA^{III}-induced transformation. These different mechanisms may be due to different methylation abilities or due to derivation of the cell lines from different species and/or different tissues. These results serve to point out how dynamic and varied the cellular response is to arsenical exposure.

ODD is predominantly linked to the initiation step of the multi-step process of carcinogenesis (Valko et al. 2006). Genomic instability and mutation is considered an “enabling characteristic” that facilitates the acquisition of the hallmarks of cancer cells (Hanahan and Weinberg 2011). Chronic arsenic exposure has recently been shown to induce the transformation of epithelial cells and decrease ROS generation in the transformed cells which, in turn, enhanced cell growth and colony formation in the transformed cells (Chang et al. 2010). Since ROS are pivotal in inducing apoptosis (Shimizu et al. 2004) their reduction once cells are transformed could help explain the enhanced cell survival in cancer cells with reduced ROS levels (Sorlie et al. 2001; Chang et al. 2010). This could also lead to an enriched population of cells containing an increased resistance to ROS and to further induction of ROS. Reduced ROS levels, partly due to an elevated level of ROS scavenging factors, is a key mechanism for resistance in normal stem cells and cancer stem cells in various tissues (Ito et al. 2004; Diehn et al. 2009). In this regard, arsenic-induced malignant transformation and tumor formation results in an overabundance of cancer stem cells (Waalkes et al. 2008; Tokar et al. 2010a, 2010c, 2011; Sun et al. 2012). Moreover, during arsenic-induced tumor formation and malignant transformation, cells show elevated levels of various oxidative stress factors, which helps the cells adapt to the metalloid (Liu et al., 2001; Pi et al., 2008; Tokar et al. 2010c; Sun et al., 2011). All together, these elements could help explain the temporal pattern of ODD seen in both cell lines during MMA^{III}-induced transformation. The early induction and increases of ODD could be acting as the “enabler” characteristic that is needed before the other cancer hallmarks (ie, anchorage-independent growth, invasion, etc.) can occur. The subsequent drop in ODD appears to occur after all the cancer cell characteristics have reached significant levels that suggest acquisition of a malignant phenotype has occurred. This drop may be the result of a reduction of induced ROS and a method by which cells avoid arsenic-induced toxicity and/or apoptosis and maintain long-term survival. It may also be the result of arsenic selecting and expanding a more stem-like population for transformation with a resultant enrichment of cancer stem cells, as has been seen in several other studies of chronic arsenic exposure (Waalkes et al.,

2008; Tokar et al. 2010a, 2010c; Sun et al. 2012). Defining the exact mechanisms involved in the observed temporal ODD pattern is beyond the scope of this current study. However, it is reasonable to suggest that some or all of these factors could be at play and, thus, warrant further investigation.

In summary, results from the present study, combined with our previous study (Kojima et al. 2009), demonstrate that arsenic methylation is not required for acquisition of a malignant phenotype. However, cellular ability to methylate arsenic may hasten and aggravate this transformation. Furthermore, the first methylation step of iAs appears to add significantly to ODD and burden as exposure of methylation-proficient cells to MMA^{III} appears not to be as good a DNA oxidant as iAs. These findings may be particularly relevant given the different polymorphisms of As3MT, the main enzyme involved in arsenic methylation, and/or the variability in the activities of different reductants between human subpopulations (Thomas et al. 2007; Engström et al. 2011). Nonetheless, taken together these results suggest that arsenicals can have both genotoxic and non-genotoxic mechanisms of action, and also serve as a 'complete carcinogen.'

Acknowledgments

The authors wish to thank Dr. Ron Mason for his expertise and advice on the immuno-spin trapping method; Drs. M. Kadiiska and Y. Sun for their critical comments and Mr. Matt Bell for assistance with figure preparation. This article may be the work product of an employee or group of employees of the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH or the United States government.

Abbreviations

ABCC	ATP binding cassette sub-family C
GST	glutathione S-transferase
HO-1	heme oxygenase-1
IST	immunospin trapping
iAs	inorganic arsenic
MMA^{III}	methylarsonous acid
ODD	oxidative DAN damage
ROS	reactive oxygen species
PTEN	phosphatase and tensin homologue
SOD	superoxide dismutase

References

- Achanzar WE, Brambila EM, Diwan BA, Webber MM, Waalkes MP. Inorganic arsenite induced malignant transformation of human prostate epithelial cells. *J Natl Cancer Inst.* 2002; 94:1888–1891. [PubMed: 12488483]
- Benbrahim-Tallaa L, Waterland RA, Styblo M, Achanzar WE, Webber MM, Waalkes MP. Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. *Toxicol Appl Pharmacol.* 2005; 206:288–298. [PubMed: 16039940]
- Bredfeldt TG, Jagadish B, Eblin KE, Mash EA, Gandolfi AJ. Monomethylarsonous acid induces transformation of human bladder cells. *Toxicol Appl Pharmacol.* 2006; 216:69–79. [PubMed: 16806342]

- Chang C, Pan J, Wang X, Zhang Z, Chen F, Shi X. Reduced reactive oxygen species-generating capacity contributes to the enhanced growth of arsenic-transformed epithelial cells. *Cancer Res.* 2010; 70:5127–35. [PubMed: 20516118]
- Cui X, Li S, Shraim A, Kobayashi Y, Hayakawa T, Kanno S, Yamamoto M, Hirano S. Subchronic exposure to arsenic through drinking water alters expression of cancer-related genes in rat liver. *Toxicol Pathol.* 2004; 32:64–72. [PubMed: 14713550]
- Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell.* 2000; 100:387–390. [PubMed: 10693755]
- Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, Qian D, Lam JS, Ailles LE, Wong M, Joshua B, Kaplan MJ, Wapnir I, Dirbas FM, Somlo G, Garberoglio C, Paz B, Shen J, Lau SK, Quake SR, Brown JM, Weissman IL, Clarke MF. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature.* 2009; 458:780–783. [PubMed: 19194462]
- Engström K, Vahter M, Mlakar SJ, Concha G, Nermell B, Raqib R, Cardozo A, Broberg K. Polymorphisms in arsenic(+III oxidation state) methyltransferase (AS3MT) predict gene expression of AS3MT as well as arsenic metabolism. *Environ Health Perspect.* 2011; 119:182–188. [PubMed: 21247820]
- Floor SL, Dumont JE, Meanhaut C, Raspe E. Hallmarks of cancer: of all cancer cells, all the time? *Trends Mol Med.* 2012; 18:509–515. [PubMed: 22795735]
- Gomez SE, del Razo LM, Munoz Sanchez JL. Induction of DNA damage by free radicals generated either by organic or inorganic arsenic (AsIII, MMAIII, and DMAIII) in cultures of B and T lymphocytes. *Biol Trace Elem Res.* 2006; 108:115–126. [PubMed: 16327065]
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: Role of ATP dependent transporters. *Nat Rev Cancer.* 2002; 2:48–58. [PubMed: 11902585]
- Hanahan D, Weinberg RA. The hallmarks of cancer: the next generation. *Cell.* 2011; 44:646–674. [PubMed: 21376230]
- Huang YC, Hung WC, Chen WT, Yu HS, Chai CY. Effects of DNMT and MEK inhibitors on the expression of RECK, MMP-9, -2, uPA and VEGF in response to arsenite stimulation in human uroepithelial cells. *Toxicol Lett.* 2011; 201:62–71. [PubMed: 21167264]
- International Agency for Research on Cancer (IARC). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. A Review of Human Carcinogens: Arsenic, Metals, Fibres, and Dusts. 2012; 100C
- Ito K, Hirao A, Arai F, Matsuoka S, Takubo K, Hamaguchi I, Nomiyama K, Hosokawa K, Sakurada K, Nakagata N, Ikeda Y, Mak TW, Suda T. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature.* 2004; 431:997–1002. [PubMed: 15496926]
- Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW. The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. *J Biol Chem.* 2000; 275:33404–33408. [PubMed: 10938093]
- Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell.* 2010; 141:53–67.
- Kitchin KT, Conolly R. Arsenic-induced carcinogenesis – oxidative stress as a possible mode of action and future research needs for more biologically based risk assessment. *Chem Res Toxicol.* 2010; 23:327–335. [PubMed: 20035570]
- Klaunig JE, Wang Z, Pu X, Zhou S. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol.* 2011; 254:86–99. [PubMed: 21296097]
- Kojima C, Ramirez DC, Tokar EJ, Himeno S, Drobna Z, Styblo M, Mason RP, Waalkes MP. Requirement of arsenic biomethylation for oxidative DNA damage. *J Natl Cancer Inst.* 2009; 101:1670–81. [PubMed: 19933942]
- Kryston TB, Georgiev AB, Pissis P, Georgakilas AG. Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res.* 2011; 711:193–201. [PubMed: 21216256]
- Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J Biol Chem.* 2003; 278:12029–12038. [PubMed: 12556532]

- Leone A, Flatow U, King CR, Sandeen MA, Marguiles IMK, Liotta LA, Steeg PS. Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. *Cell*. 1991; 65:25–35. [PubMed: 2013093]
- Leslie EM, Haimeur A, Waalkes MP. Arsenic transport by the human multidrug resistance protein (MRP1/ABCC1): evidence that a tri-glutathione conjugate is required. *J Biol Chem*. 2004; 279:32700–32708. [PubMed: 15161912]
- Li G, Lee LS, Li M, Tsao SW, Chiu JF. Molecular changes during arsenic-induced cell transformation. *J Cell Physiol*. 2011; 226:3225–3232. [PubMed: 21344382]
- Liu J, Chen H, Miller DS, Saavedra JE, Keefer LK, Johnson DR, Klaassen CD, Waalkes MP. Overexpression of glutathione S-transferase II and multidrug resistant transport proteins is associated with acquired arsenic tolerance to inorganic arsenic. *Mol Pharmacol*. 2001; 60:302–309. [PubMed: 11455017]
- Mass MJ, Tennant A, Roop BC, Cullen WR, Styblo M, Thomas DJ, Kligerman AD. Methylated trivalent arsenic species are genotoxic. *Chem Res Toxicol*. 2001; 14:355–361. [PubMed: 11304123]
- McKenzie S, Kyprianou N. Apoptosis evasion: the role of survival pathways in prostate cancer progression and therapeutic resistance. *J Cell Biochem*. 2006; 97:18–32. [PubMed: 16216007]
- Ming M, He Y-Y. PTEN in DNA damage repair. *Cancer Lett*. 2012; 319:125–129. [PubMed: 22266095]
- National Toxicology Program (NTP). 12th Report on Carcinogens. Department of Health and Human Services; Research Triangle Park, NC: 2011.
- Nesnow S, Roop BC, Lambert G, Kadiiska M, Mason RP, Cullen WR, Mass MJ. DNA damage induced by methylated trivalent arsenicals is mediated by reactive oxygen species. *Chem Res Toxicol*. 2002; 15:1627–1634. [PubMed: 12482246]
- Paul DS, Harmon AW, Devesa V, Thomas DJ, Styblo M. Molecular mechanisms of the diabetogenic effects of arsenic: inhibition of insulin signaling by arsenite and methylarsonous acid. *Environ Health Perspect*. 2007; 115:734–742. [PubMed: 17520061]
- Pi J, Diwan BA, Sun Y, Liu J, Qu W, He Y, Styblo M, Waalkes MP. Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. *Free Radic Biol Med*. 2008; 45:651–658. [PubMed: 18572023]
- Ramirez DC, Mejiba SE, Mason RP. Immuno-spin trapping of DNA radicals. *Nat Methods*. 2006; 3:123–127. [PubMed: 16432522]
- Ramirez DC, Gomez-Mejiba SE, Mason RP. Immuno-spin trapping analysis of DNA radicals. *Nat Protoc*. 2007; 2:512–522. [PubMed: 17406615]
- Romach EH, Zhao CQ, Del Razo LM, Cebrian ME, Waalkes MP. Studies on the mechanisms of arsenic-induced self tolerance developed in liver epithelial cells through continuous low-level arsenite exposure. *Toxicol Sci*. 2000; 54:500–508. [PubMed: 10774833]
- Schwerdtle T, Walter I, Mackiw I, Hartwig A. Induction of oxidative DNA damage by arsenite and its trivalent and pentavalent methylated metabolites in cultured human cells and isolated DNA. *Carcinogenesis*. 2003; 24:967–974. [PubMed: 12771042]
- Shimizu T, Numata T, Okada Y. A role of reactive oxygen species in apoptotic activation of volume-sensitive Cl(–) channel. *Proc Natl Acad Sci*. 2004; 101:6770–6773. [PubMed: 15096609]
- Sørli T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE, Børresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci*. 2001; 98:10869–10874. [PubMed: 11553815]
- Stueckel TA, Lu Y, Davis ME, Wang L, Jiang BH, Holaskova I, Schafer R, Barnett JB, Rojanasakul Y. Chronic occupational exposure to arsenic induces carcinogenic gene signaling networks and neoplastic transformation in human lung epithelial cells. *Toxicol Appl Pharmacol*. 2012; 261:204–216. [PubMed: 22521957]
- Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Arch Toxicol*. 2000; 74:289–299. [PubMed: 11005674]

- Sun Y, Kojima C, Chignell C, Mason RM, Waalkes MP. Arsenic transformation predisposes human skin keratinocytes to UV-induced DNA damage yet enhances their survival apparently by diminishing oxidant response. *Toxicol Appl Pharmacol.* 2011; 255:242–250. [PubMed: 21820459]
- Sun Y, Tokar EJ, Waalkes MP. Overabundance of putative cancer stem cells in human skin keratinocyte cells malignantly transformed by arsenic. *Toxicol Sci.* 2012; 125:20–29. [PubMed: 22011395]
- Thomas DJ, Li J, Waters SB, Xing W, Adair BM, Drobna Z, Devesa V, Styblo M. Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals. *Exp Biol Med (Maywood).* 2007; 232:3–13. [PubMed: 17202581]
- Tokar EJ, Webber MM. Cholecalciferol (vitamin D3) inhibits growth and invasion by up-regulating nuclear receptors and 25-hydroxylase (CYP27A1) in human prostate cancer cells. *Clin Exp Metastasis.* 2005; 22:275–284. [PubMed: 16158255]
- Tokar EJ, Diwan BA, Waalkes MP. Arsenic exposure transforms human epithelial stem/progenitor cells into a cancer stem-like phenotype. *Environ Health Perspect.* 2010a; 118:108–115. [PubMed: 20056578]
- Tokar EJ, Benbrahim-Tallaa L, Ward JM, Lunn R, Sams RL 2nd, Waalkes MP. Cancer in experimental animals exposed to arsenic and arsenic compounds. *Crit Rev Toxicol.* 2010b; 40:912–27. [PubMed: 20812815]
- Tokar EJ, Qu W, Liu J, Liu W, Webber MM, Phang JM, Waalkes MP. Arsenic-specific stem cell selection during malignant transformation. *J Natl Cancer Inst.* 2010c; 102:638–649. [PubMed: 20339138]
- Tokar EJ, Diwan BA, Ward JM, Delker DA, Waalkes MP. Carcinogenic effects of “whole-life” exposure to inorganic arsenic in CD1 mice. *Toxicol Sci.* 2011; 119:73–83. [PubMed: 20937726]
- Tokar EJ, Diwan BA, Thomas DJ, Waalkes MP. Tumors and proliferative lesions in adult offspring after maternal exposure to methylarsonous acid during gestation in CD1 mice. *Arch Toxicol.* 2012; 86:975–982. [PubMed: 22398986]
- Valko M, Rhodes CJ, Moncol J, Isakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact.* 2006; 160:1–40. [PubMed: 16430879]
- Waalkes MP, Liu J, Diwan BA. Transplacental arsenic carcinogenesis in mice. *Toxicol Appl Pharmacol.* 2007; 222:271–280. [PubMed: 17306315]
- Waalkes MP, Liu J, Germolec DR, Trempus CS, Cannon RE, Tokar EJ, Tennant RW, Ward JM, Diwan BA. Arsenic exposure in utero exacerbates skin cancer response in adulthood with contemporaneous distortion of tumor stem cell dynamics. *Cancer Res.* 2008; 68:8278–8285. [PubMed: 18922899]
- Wnek SM, Jensen TJ, Severson PL, Futscher BW, Gandolfi AJ. Monomethylarsonous acid produces irreversible events resulting in malignant transformation of a human bladder cell line following 12 weeks of low-level exposure. *Toxicol Sci.* 2010; 116:44–57. [PubMed: 20375083]
- Wnek SM, Kuhlman CL, Camarillo JM, Medeiros MK, Liu KJ, Lau SS, Gandolfi JA. Interdependent genotoxic mechanisms of monomethylarsonous acid: role of ROS-induced DNA damage and poly(ADP-ribose) polymerase-1 inhibition in the malignant transformation of urothelial cells. *Toxicol Appl Pharmacol.* 2011; 257:1–13. [PubMed: 21925530]
- Xu Y, Tokar EJ, Sun Y, Waalkes MP. Arsenic-transformed malignant prostate epithelia can convert noncontiguous normal stem cells into an oncogenic phenotype. *Environ Health Perspect.* 2012; 120:865–871. [PubMed: 22472196]
- Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP. Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proc Natl Acad Sci.* 1997; 94:10907–10912. [PubMed: 9380733]

Figure 1.

Activity of secreted matrix metalloproteinase (MMP)-2 and -9 in (A) liver and (B) prostate cells chronically exposed to MMA^{III}. Both cell lines were exposed to 1 μ M MMA^{III} for 30 weeks, and MMP-2 and -9 in conditioned media were measured by SDS-PAGE zymography. Time-dependent increases in MMP activity were seen in both cell lines starting at 15 weeks of MMA^{III} exposure. Data represent mean $\pm$ SEM ($n = 3$). Asterisk (*) indicates statistical significance compared with time-matched untreated controls, $p < 0.05$.

Figure 2.

Colony formation in soft agar and invasion during chronic exposure to MMA^{III} in liver and prostate cells. (A) Liver cells started showing a time-dependent increase at 5 weeks and (B) prostate cells started showing a time-dependent increase in colony formation at 15 weeks of MMA^{III} exposure. (C, D) Both cell lines showed very similar time-dependent increases in invasion starting at 15 weeks of MMA^{III} exposure. Data are expressed as mean $\pm$ SEM ($n = 3$). Asterisk (*) indicates statistical significance compared with time-matched untreated controls, $p < 0.05$.

Figure 3. PTEN levels during chronic MMA^{III} exposure of liver and prostate cells. (A, B) Transcript levels for *PTEN* show a similar depletion starting at 10 weeks in both the (A) liver and (B) prostate cell lines. Data are expressed as mean $\pm$ SEM (n = at least 3). Asterisk (*) indicates statistical significance compared with time-matched untreated controls, $p < 0.05$.

Figure 4. Oxidative DNA damage (ODD) during chronic MMA^{III} exposure. Methylation-proficient liver cells (A) and methylation-deficient prostate cells (B) were exposed to various concentrations of MMA^{III} for 30 weeks, and ODD was assessed by the formation of DNA–nitronne adducts using the immuno-spin trapping method. Strikingly similar temporal patterns of ODD were seen between the methylation-proficient and methylation-deficient cells. Data are expressed as mean $\pm$ SEM (n = 9).

Figure 5.

Direct comparison of ODD peaks (at around 20 weeks of exposure; from Figure 4) in cells exposed to different concentrations of MMA^{III} . Peak ODD levels at the highest MMA^{III} concentration (1 μM) were similar between the two cell lines but at the lower concentrations (0.25 μM and 0.5 μM) tended to be somewhat lower in the methylation-proficient liver cells compared with the methylation-deficient prostate cells. Data were taken from Figure 4.

Figure 6.

Comparison of MMP activity and ODD levels induced by exposure to iAs and MMA^{III}. MMP activity and ODD levels in the methylation-deficient prostate cells (A, B) and methylation-proficient liver cells (C, D) exposed to iAs and MMA^{III} at the approximate point of transformation for each arsenical. Asterisk (*) indicates statistical significance compared with time-matched, arsenical-matched untreated controls, $p < 0.05$. Data for iAs taken from Kojima et al. 2009.

Figure 7. Levels of arsenical efflux-associated factors. Transcript levels of *GST-π*, *ABCC1*, and *ABCC2* were measured in prostate (A) and liver (B) cells during chronic MMA^{III} (1 μM) exposure for 30 weeks. Data are expressed as mean ± SEM (n = 3). Asterisk (*) indicates statistical significance compared with time-matched untreated controls, $p < 0.05$.

Figure 8. Levels of oxidative stress-associated factors. Transcript levels of *NRF2*, *SOD-1*, *SOD-2*, and *HO-1* were measured in prostate (A) and liver (B) cells during chronic MMA^{III} (1 μ M) exposure for 30 weeks. Data are expressed as mean $\pm$ SEM ($n = 3$). Asterisk (*) indicates statistical significance compared with time-matched untreated controls, $p < 0.05$.