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Metallothionein Blocks Oxidative DNA Damage Induced by Acute Inorganic Arsenic Exposure

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Abstract

We studied how the protein metallothionein (MT) impacts arsenic-induced oxidative DNA damage (ODD) using cells that poorly express MT (MT-I/II double knockout embryonic cells; called MT-null cells) and wild-type (WT) MT competent cells. Arsenic (as NaAsO₂) was less cytotoxic over 24 h in WT cells (LC₅₀ = 11.0 ± 1.3 μM; mean ± SEM) than in MT-null cells (LC₅₀ = 5.6 ± 1.2 μM). ODD was measured by the immuno-spin trapping method. Arsenic (1 or 5 μM; 24 h) induced much less ODD in WT cells (121% and 141% of control, respectively) than in MT-null cells (202% and 260%). In WT cells arsenic caused concentration-dependent increases in MT expression (transcript and protein), and in the metal-responsive transcription factor-1 (MTF-1), which is required to induce the MT gene. In contrast, basal MT levels were not detectable in MT-null cells and unaltered by arsenic exposure. Transfection of MT-I into the MT-null cells markedly reduced arsenic-induced ODD levels. The transport genes, *Abcc1* and *Abcc2* were increased by arsenic in WT cells but either showed no or very limited increases in MT-null cells. Arsenic caused increases in oxidant stress defense genes *HO-1* and *GSTa2* in both WT and MT-null cells, but to much higher levels in WT cells. WT cells appear more adept at activating metal transport systems and oxidant response genes, although the role of MT in these responses is unclear. Overall, MT protects against arsenic-induced ODD in MT competent cells by potentially sequestration of scavenging oxidant radicals and/or arsenic.

Keywords

Arsenic; oxidative DNA damage; metallothionein; reactive oxygen species

Introduction

Arsenic, a metalloid with some carbon-like properties, is ubiquitous in the environment. Significant exposure to inorganic arsenic can occur in human populations, especially

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CONFLICT of INTEREST STATEMENT

None.

through contaminated drinking water (IARC, 2012). Inorganic arsenic is a multisite human carcinogen (IARC, 2012). However, the molecular mechanisms of arsenic carcinogenesis remain unclear. Some studies link arsenic exposure to the production of reactive oxygen species (ROS) and indicate ROS-mediated oxidative DNA damage (ODD) is a potential carcinogenic mechanism (Nesnow et al., 2002; Valko et al., 2005; Kojima et al., 2009; Jomova et al., 2011). It has been proposed that in some cases ROS plays a role in arsenic carcinogenesis, such that excess ROS overwhelms the antioxidant capacity of a target cell and results in molecular damage to critical macromolecules, most significantly DNA (Jomova et al., 2011). In this regard, ODD is a hallmark of damage produced by various carcinogenic metals and can initiate the carcinogenic process (Jomova and Valko, 2011). Inorganic arsenic-induced ODD certainly occurs in some arsenic target relevant cell types and can be correlated with malignant transformation *in vitro* (Kojima et al., 2009). It does not occur, however, in all cells exposed to survivable levels of inorganic arsenic undergoing *in vitro* transformation (Kojima et al., 2009).

Metallothionein (MT) is a low-molecular weight, cysteine-rich and metal-binding family of proteins (Klaassen et al. 2009). The major isoforms of the MT gene, MT-I and MT-II, are common in mammalian cells and inducible by various inorganics, including arsenic (Haq et al., 2003; He and Ma, 2009). MT-III and MT-IV are considered minor MT forms with limited distributions. MT-III is located primarily in the brain and MT-IV is located in stratified squamous epithelia of the gastro-intestinal tract (Laity and Andrews, 2007). MT plays an important role in the homeostasis of essential metals, like zinc, and in detoxification of inorganics like cadmium (Klaassen et al., 2009). It is clear that inorganic arsenic can bind to MT (Irvine et al., 2013; Irvine and Stillman, 2013) and MT mitigates arsenic toxicity both *in vivo* and *in vitro* (Liu et al., 2000; Miao et al., 2013). MT can limit ODD induced by other inorganic carcinogens, like cadmium (Qu et al., 2013). Although MT can mitigate arsenic toxicity (Liu et al., 2000; Miao et al., 2013), how MT might impact arsenic-induced ODD has not been well defined. Like inorganic arsenic, ROS and oxidative stress also induce MT expression (Qu et al., 2009; 2013; Braithwaite et al., 2010). Since MTs contain large amounts of thiol groups, they also appear to act as antioxidants and can protect against oxidative stress (Bell and Vallee, 2009), as the multiple cysteines may react directly with oxidants. As arsenicals can produce ODD via ROS (Jomova et al., 2011; Kojima et al., 2009; Tokar et al., 2014), it is reasonable to hypothesize that cellular MT would mitigate arsenic-induced ODD in cells where the metalloid produces ODD. However, the role of MT in arsenic-induced ODD is not completely defined. In human populations, persons that show a poor ability to express MT, as reflected in low bloodborne MT transcript levels, appear predisposed to arsenicosis and associated precancerous skin lesions (Liu et al., 2007). Similarly, MT deficiency causes a general hypersensitivity to skin carcinogenesis induced by organic carcinogens (Suzuki et al., 2003).

Metal-activated transcription factor 1 (MTF-1) is a multipotent regulator of transcription often involved in the adaptation to stress (Günther et al., 2012). MTF-1 coordinates transcriptional regulation of MTs, metal transporters, and various antioxidant proteins (He and Ma, 2009). Arsenic-induced MTF-1 binds to the metal response elements of the *MT-I* gene *in vitro* thereby inducing *MT-I* mRNA (He and Ma, 2009). Others have found that *MT-*

I, *MT-II* and *MT-III* can all be induced by arsenic exposure *in vitro* (Falnoga et al., 2012). Similarly, the ATPbinding cassette (ABC) transporter proteins, multidrug resistance protein 1 (*ABCC1*) and *ABCC2*, play key roles in cellular arsenic efflux and detoxification by pumping arsenicglutathione (GSH) conjugates out of the cell (Leslie, 2012). GSH is a primary intracellular antioxidant and bio-conjugating compound and plays a major role as a substrate for glutathione transferase (GST) to protect cells against oxidants (Cuyper et al., 2010). Glutathione Stransferase- α 2 (GST α 2) is one of the main isoforms of the GST family. Similarly, heme oxygenase 1 (HO-1) is an antioxidant enzyme that can protect cells from oxidative stress (Jiang et al., 2014). Antioxidant genes, such as HO-1 and GST α 2, work together with ABCC transport proteins to reduce arsenic toxicity (Kala et al., 2000; Leslie et al., 2004; Tokar et al., 2014). All of these factors can help reduce arsenic-induced oxidative damage and toxicity. However, the potential involvement of MT in these processes is unclear.

Thus, the purpose of this study was to determine if MT protects from acute inorganic arsenic-induced ODD. As a test system, we selected embryonic cells with knocked-out MT-I and MT-II (MT-null) cells and compared responses to phenotypically normal wild type (WT) cells.

Material and methods

Chemicals and reagents

Sodium *m*-arsenite (NaAsO₂) was purchased from Sigma (St. Louis, MO). The monoclonal mouse anti-MT antibody obtained from Dako Corporation (Carpinteria, CA) reacts well with both MT-I and MT-II protein. The spin trap reagent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Alexis Biochemicals, San Diego, CA) was purified twice by vacuum sublimation at 15–25°C and stored under argon atmosphere at –80 °C until use. The cell Titer 96 Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Madison, WI).

Cell culture

A cell line created from the embryonic cells of transgenic mice with targeted disruption of the MT-I/II genes, termed MT-null cells, and a WT control cell line from the parental strain of mice were kindly provided by Dr. John Lazo, University of Pittsburgh (Lazo et al. 1995). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere as described previously (Lazo et al. 1995).

Cytotoxicity assay

The acute cytotoxicity of inorganic arsenic was defined by metabolic integrity using cell proliferation assay kits as previous described (Qu et al, 2002). After media containing several concentrations (0, 1, 5, 10, 15, 20, 25, or 50 μ M) of inorganic arsenic were added, cells were incubated for 24 h and viability was determined. The values for the lethal concentration in 50% of the cells (LC₅₀) values were defined from analysis of the linear portion of four separately derived metabolic integrity curves.

MT transfection into MT-null cells

MT-null cells were made stably MT-competent via transfection of the MT-I gene using a method described in detail in prior work (Zuo et al. 2009). In brief, complexes between pcDNA3-MT and the FuGen 6 Transfection Reagent (Roche, Mannheim, Germany) were prepared to the specifications of the manufacturer using the MT-I gene. The expression of MT-I was driven by a constitutive vector promoter CMV. After pcDNA3-MT-reagent complexes had formed, the mixture was exposed to cultures and incubated for 16 h before replacing the Dulbecco's modified Eagle's medium. FuGen 6 transfection system was used according to guidelines for 6-well plates with a volume of 3 µl FuGen 6 per well. These MT-null transfected with MT-I cells are defined as stably transfected with the gene in previous work (Zuo et al., 2009).

Oxidative DNA damage measurement

ODD was measured by the Immuno-spin trapping (IST) method (Ramirez et al., 2006; Ramirez et al., 2007). The IST method measures cellular DNA radicals by *in situ* reaction with DMPO causing the conversion of the radicals to stable DMPO-nitron adducts. This is followed by subsequent isolation of the DNA and immunochemical quantitation via ELISA of the nitron adducts using a primary rabbit anti-DMPO polyclonal antibody and a goat anti-rabbit secondary antibody conjugated to horse-radish peroxidase (Kojima et al., 2009). Since this method fixes DNA radicals before isolation, it avoids artifacts potentially introduced during cell disruption and DNA isolation and markedly reduces background signals (Ramirez et al., 2006; Ramirez et al., 2007). This likely is of particular importance when working with arsenic which could be released from loose binding sites by this processing and cause artifactual oxidative damage during DNA isolation.

Analysis of gene expression at the transcriptional level

Total RNA was isolated from cells using TRIzol (GIBCO/BRL Life Technologies) and then subjected to DNase digestion using the RNase-Free DNase Set (Qiagen, Valencia, CA) followed by cleanup using the RNeasy Mini kit (Qiagen, Valencia, CA). The resultant DNA-free RNA was quantitated by UV spectroscopy at 260 nm and stored in RNase-free H₂O at -80°C. Quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) was conducted as described previously (Zuo et al., 2009). The primers were designed using Primer Express software (Applied Biosystems) and included: *MT-I* : Forward 5'- AAT GTG CCC AGG GCT GTG T-3'; Reverse, 3'-GCT GGG TTG GTC CGA TAC TAT T-5'; *MT-II* : Forward 5'- TGT GCC TCC GAT GGA TCC T-3'; Reverse, 3'-GCA GCC CTG GGA GCA CTT-5'; *Abcc1* : Forward 5'-TGG TGA CAG ACA CCG TAG GAA A-3'; Reverse 3'-TGT GTT GCT GGC TGG TAT CC-5'; *Abcc2* : Forward 5'- TGC AGC TTC CTT GAC CAT GA-3'; Reverse 3'- CCT GCT GCC GGA CCT AGA G-5'; *Mtf-1* : Forward 5'- GCC CCT GCC TGC TAT GGT-3'; Reverse 3'- AGG ATT GCC GTG TTA GGA GTT G-5'; *HO-1* : Forward 5'-CCT CAC TGG CAG GAA ATC ATC-3'; Reverse 3'- CCT CGT GGA GAC GCT TTA CAT A-5'; *GST-α2* : Forward 5'- CTT GTT GGG CCC CAC ATC T-3'; Reverse 3'- CTG GGA TGC CCT TCA AAG ACT-5'; β -*actin* : Forward 5'-GGC CAA CCG TGA AAA GAT GA-3'; Reverse 3'-CAG CCT GGA TGG CTA CGT ACA-5'.

Analysis of gene expression at the translational level

Cells were lysed by adding 1X sodium dodecyl sulfate lysis buffer containing 1% Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktail 1 (Sigma, St. Louis, MO). The cells were then scraped off the plate and transferred to a microcentrifuge tube on ice. The samples were sonicated for 10–15 seconds to shear DNA and reduce viscosity, and then centrifuged at 14,000 Xg for 10 min. The resulting supernatants (termed cytosol) were used for determining MT protein levels by Western blot (20 µg total protein loaded per sample). Relative densities of the bands were digitally quantified by using Bio-Rad Quantity One-4.4.0 analysis software (Qu et al. 2013).

Statistical analysis

Data are expressed as mean ± SEM of four or more replicates. A Student's t-test was used when directly comparing the responses of the two different cell lines (LC₅₀) at a given treatment level. An ANOVA with subsequent Dunnett's test was used when comparing a concentration-dependent response to arsenic within a single cell line to its specific control. When comparing responses at multiple concentrations in two cell lines, a Duncan's test was used after ANOVA which compares all groups to each other. When comparing response in three cell lines, a Duncan's test was also used after ANOVA. Differences were considered significant at a level of two-sided $p < 0.05$.

Results

The presence of MT reduces inorganic arsenic-induced cytotoxicity and ODD

MT-null and WT cells were treated with various levels of inorganic arsenic (0, 1, 5, 10, 15, 20, 25, or 50 µM) for 24 h and cytolethality was measured. The arsenic was much less cytolethal in MT-competent WT cells than in the MT-null cells as reflected in the fact that LC₅₀ for arsenic was nearly 2-fold greater in WT cells than that in MT-null cells (Fig. 1A). To determine if the presence of MT reduces ODD, MT-null and WT cells were exposed to inorganic arsenic (1 or 5 µM) and ODD was assessed. All ODD data are normalized in both cell types to control equals 100% but baseline ODD between control WT and MT-null were not remarkable. ODD showed a marked, concentration-dependent increase in arsenic-exposed MT-null cells. In WT cells, ODD was modestly increased but only at the highest inorganic arsenic level (Fig. 1B).

Analysis of MT related gene expression

MT-null and WT cells were exposed to various concentrations (1 or 5 µM) of arsenic for 24 h and the expression of MT and various related genes was assessed. In WT cells, arsenic induced concentration-dependent increases in the expression of *MT-I*, *MT-II* transcript (Fig. 2A, 2B) and MT I/II protein (Fig. 2C). In MT-null cells, *MT-I* and *MT-II* mRNA (not shown) as well as MT protein (Fig. 2C; inset) were undetectable. Arsenic increased the expression of *Mtf-1* transcript in WT cells at both concentrations but only at the highest concentration in MT-null cells (Fig. 2D). It is unclear why deletion of MT-I and MT-II would compromise the induction of *mtf-1* by arsenic (Fig. 2D). MT in untreated normal cells generally represents a pool of zinc and since MTF-1 activation is very sensitive to zinc,

including zinc displaced from MT by other metals (Laity and Andrews, 2007; Okumura et al., 2011), the absence of the major forms of MT in the MT-null cells may eliminate a major potential stimulator of MTF-1 production.

Expression of efflux and oxidant stress response genes after arsenic exposure

Enhanced metal transport may be an adaptive response to arsenic. Thus, expression of two genes that can function in arsenic efflux, namely *Abcc1* and *Abcc2* were examined after arsenic treatment. Arsenic increased *Abcc1* and *Abcc2* transcripts in a concentration-dependent manner in WT cells (Fig. 3A and B). In MT-null cells, arsenic increased expression of *Abcc2* but only at the highest concentration (Fig. 3B). The levels of *Abcc2* induced in MT-null cells exposed to 5 μ M arsenic were still much less compared to similarly-treated WT cells.

MT-null and WT cells were also tested for expression of oxidant stress response genes after arsenic exposure. Arsenic caused concentration-dependent increases in *HO-1* (Fig. 4A) and *GST- α 2* (Fig. 4B) transcripts, two important oxidant stress genes, in both WT and MT-null cells. However, the response in WT cells was much more robust than in MT-null cells.

MT transfection into MT-null cells protects against arsenic

To determine whether MT expression is a key factor in protection against inorganic arsenic-induced ODD and oxidative stress response, MT-null cells were first transfected with the MT-I gene, then MT-null, WT and MT-null transfected with MT-I cells were exposed to inorganic arsenic for 24 h. Basal MT-I protein levels subsequently became easily detectable in the MT-null cells transfected with MT-I like WT cells (Fig. 5A), but remained undetectable in MT-null cells (not shown). MT-null cells transfected with MT-I showed markedly increased basal *MT-I* expression, indicating the transfection of the gene was successful, and when exposed to inorganic arsenic showed increased *MT-I* transcript (Fig. 5B). In MT-null cells transfected with MT-I gene the LC₅₀ of arsenic was increased compared to MT-null cells, indicating arsenic was less toxic in the MT-null transfected with MT-I cells (Fig. 5C). Similarly, arsenic induced less ODD in MT-null transfected with MT-I cells than that in MT-null cells (Fig. 5D). Thus, transfection of the MT-I gene into MT-null cells made them resistant to arsenic-induced cytotoxicity and ODD.

Discussion

Although arsenic is clearly a human carcinogen, the mechanisms by which arsenic induces cancer remain unclear. A number of studies indicate that inorganic arsenic exposure can induce the generation of free radicals in cells (Shi et al., 2004). Arsenic can also increase the levels of ROS which play an important causal role in the genotoxicity of arsenical compounds at least in some cells (Liu et al., 2001; Shi et al., 2004). Oxidant stress and resultant ROS are thought to be involved in the damage of critical cell molecules, such as DNA, and it is thought oxidant stress may be linked with the development of arsenic-induced carcinogenesis (Valko et al., 2005). Thus, arsenic-induced oxidative damage may be one underlying mechanism for arsenic carcinogenesis (Hei et al., 1998; Valko et al., 2005), although this does not appear to be the case in all target cells (Kojima et al., 2009).

Arsenic compounds can induce overexpression of key genes relative to cancer (IARC, 2012), including HO-1, an oxidative stress protein (Wang et al., 2012). It is clear that arsenic does not work exclusively through genotoxic mechanisms and may well have multiple mechanisms depending on the target cell in question (Kojima et al., 2009). Prior work shows that cells that do not metabolize inorganic arsenic to its methylated forms do not show ODD even as they undergo arsenic-induced malignant transformation (Kojima et al., 2009). As another factor in the eventual extent of DNA damage, in the present study it is clear that cells with a strong ability to produce MT showed little sensitivity to inorganic arsenic induced ODD.

It is clear that MT can bind inorganic arsenic (Irvine et al., 2013; Irvine and Stillman, 2013) and this includes arsenic binding to human MT (Ngu and Stillman, 2006). Persons that poorly express MT are more prone to arsenic-induced toxic effects, as in early skin lesions (Liu et al., 2007), while MT can prevent cell death or toxicity *in vitro* (e.g. Miao et al., 2013) and *in vivo* in mice (Liu et al., 2000). It is, however, very hard to distinguish between mechanisms that involves MT sequestration of the metalloid or MT acting as an antioxidant that sequesters arsenic-generated ROS. Indeed, pre-treatment of mice with a dose of zinc that increases liver MT 150-fold over baseline protected against the subsequent acute lethal effects of arsenite (Kreppel et al., 1994). However, in this one study little arsenic was found to be bound to purified liver MT after zinc-induction and arsenic exposure (Kreppel et al., 1994). This would indicate *in vivo* arsenic may only poorly displace endogenous metals, like zinc, from MT. In fact the authors found that tissue dosimetry of arsenic indicated more rapid arsenic elimination was a major factor in acquired tolerance (Kreppel et al., 1994). This does not eliminate the possibility that some arsenic could be bound to MT that the metalloid induced in the present study however, given its ability to bind to metal-free MT (Ngu and Stillman, 2006). Indeed, with our study on the MT-null cells transfected with MT-I, MT-I expression is driven by a constitutive vector promoter, CMV, and not its native promoter (see Methods) so the increased expression after arsenic exposure in these cells would likely be indirect. The increased basal MT in MT-null cells transfected with MT-I would very likely represent a pool of potentially displaceable zinc and zinc displaced from MT has been implicated in CMV promoter-driven expression (Kanekiyo et al., 2000).

MT has a high cysteine content, which makes it a potentially efficient scavenger of ROS (Klaassen et al., 2009; Qu et al., 2013). Thus, MT can protect cells from oxidative damage by directly scavenging ROS (Braithwaite et al., 2010). Increased MT expression will protect cells from oxidative stress and ROS-mediated damage (Bell and Vallee, 2009; Klaassen et al., 2009; Qu et al., 2013) and MT-null cells are hypersensitive to ROS-generating toxicants like cancer chemotherapeutics (Lazo et al., 1995). It was evident that MT-competent WT cells were more resistant to acute arsenic compared to MT-null cells in the present study. This is consistent with findings that MT-transgenic mice that over-express MT when compared to MT-null mice, are less sensitive to the hepatotoxic and nephrotoxic effects of chronic oral or injected inorganic arsenicals (Liu et al., 2000; Klaassen et al., 2009). Similarly, it has been shown that cadmium and hydrogen peroxide (H₂O₂), a direct oxidant, are much more lethal to MT-null cells than WT cells (Qu et al., 2013) and MT-null mice are sensitive to liver cancers induced by either cadmium (Waalkes and Liu, 2009) or cisplatin

(Waalkes et al., 2006) compared to WT mice. Cadmium can cause oxidative stress in an indirect fashion (Waalkes, 2003; Qu et al., 2013). Cisplatin is thought to be toxic in many cases via oxidant stress (Pabla and Dong, 2008) and like cadmium, can be bound by MT (Knipp 2009). Thus, it appears at least part of the capacity of MT to mitigate arsenic toxicity and ODD seen in the present study is due to its ability to mitigate oxidative stress potentially via scavenging arsenic-induced ROS. It has been shown that arsenite or its methylated metabolites can induce ODD *in vitro* (Jomova and Valko, 2011; Liu et al., 2021; Kojima et al., 2009; Tokar et al., 2014), depending on the cell type. The fact that transfection of MT-I into MT-null cells in the present work blocked arsenic-induced ODD points to a direct role for MT protein in reducing ODD formation, and indicates an oxidant sequestration mechanism is primary at play, although some sequestration by MT of arsenic could have occurred. The activation of oxidant stress response genes (HO-1, GST α 2, ABCC-1 and ABCC-2) in WT cells after arsenic exposure also argues for reaction to oxidative stress as a primary response, rather than reaction to arsenic levels. The fact that MT-null cells did not compensate for the lack of MT by upregulating other stress response genes (HO-1, GST, etc.) more than WT cells in the present study may seem counter-intuitive. However, *in vivo* when MT-null (MT-I/II knockout) mice are exposed to various chemical or physical oxidative stressors they do not compensate for the reduced MT levels by increasing other known oxidant defense systems (Chiaverini and De Ley, 2010). The reasons for this are not exactly clear but it may be that MT is central in activating these defense systems. In any event, MT appears to confer a generalized resistance to arsenic-induced oxidant stress and attack on DNA. The fact that MT was rapidly up-regulated in response to arsenic in WT cells in the present study and by cadmium and H₂O₂ in prior work (Qu et al., 2013) shows this is a dynamic response system to oxidative stress.

Arsenic apparently precipitated expression of MT via MTF-1 (He and Ma, 2009). MTF-1 will coordinate the transcriptional regulation of MT as well as metal transporters and various antioxidant proteins, all of which can play important roles in protection against the toxicity of metals (He and Ma, 2009). The present results show that arsenic increased expression of *Mtf-1* and MT in WT cells, supporting previous data that indicates MTF-1 mediates the induction of MT by arsenic (He and Ma, 2009). *Abcc1* and *Abcc2* genes encode for the efflux transporter ABCC1 and ABCC2 proteins that function as cell membrane pumps. ABCC1 and ABCC2 proteins protect cells from inorganic and methylated arsenic species through the efflux of arsenic-GSH conjugates and are crucial for cellular arsenic elimination (Leslie, 2012). ABCC1 decreases the cytotoxicity of arsenic in several cell lines (Vernhet et al., 2001; Liu et al., 2001; Leslie et al., 2004; Kojima et al., 2006). In the present study, arsenic increased expression of both *Abcc1* and *Abcc2* in WT cells while only at the highest concentration of arsenic did it increase *Abcc2* expression in MT-null cells and then only modestly. This suggests that in MT competent cells there is a link between MT, efflux proteins and arsenic tolerance. In our previous study H₂O₂ exposure also increased expression of *Abcc1* and *Abcc2* in WT cells, but not MT-null cells (Qu et al., 2013), again suggesting these genes are activated as part of a coordinated adaptive oxidative stress process in MT competent cells. In fact, ABCC1 and ABCC2 can act synergistically with several phase II conjugating enzymes, like GST, to confer resistance to drugs and carcinogens (Smitherman et al., 2004; Morrow et al., 2006), including arsenic (Leslie et al.,

2004). In the present study, we also found that arsenic stimulated greater expression of antioxidant genes such as *HO-1* and *GST-α2* in WT cells than MT-null cells. Exactly how these cellular defense mechanisms are activated during arsenic exposure and how they are related with MT gene remains to be elucidated.

In summary, the present work suggests that MT protects against arsenic-induced ODD possibly by multiple mechanisms, including sequestration of arsenic-induced oxidant species and perhaps direct arsenic sequestration. Activation of MTF-1, metal transporters and anti-oxidative proteins also helps protect from the oxidative damage induced by the metalloid. MT-competent cells are more adept at activating metal transport systems and oxidant response genes, although the role of MT in these responses is unclear.

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Abbreviations

ABCC1	Multidrug resistance-related protein 1
ABCC2	Multidrug resistance-related protein 2
As	Arsenic
DMPO	5,5-dimethyl-1-pyrroline N-oxide
GST	Glutathione S-transferase
GSTα2	Glutathione S-transferase-α2
HO-1	Heme oxygenase 1
H₂O₂	Hydrogen peroxide
IST	Immuno-spin trapping
LC₅₀	Lethal concentration 50%
MT	Metallothionein
MTF-1	Metal-responsive transcription factor 1
MT-null	MT-I/II knockout
ODD	Oxidative DNA damage
RT-PCR	Reverse transcription-polymerase chain reaction
ROS	Reactive oxygen species
WT	Wild type

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Potential Highlights

Metallothionein blocks arsenic toxicity

Metallothionein reduces arsenic-induced DNA damage

Metallothionein may bind arsenic or radicals produced by arsenic

Figure 1. MT protects against arsenic-induced cytolethality and ODD

MT-null or WT cells were exposed to various concentrations of inorganic arsenic for 24 h, cytolethality and ODD were assessed. A) Mean LC₅₀ for arsenic in WT and MT-null cells. An asterisk (*) indicates a significant difference from WT by Student's t-test. B) Dose-response of arsenic and ODD in WT and MT-null cells. Values represent the mean $\pm$ SEM (n = 4). In figure 1B, an asterisk indicates a significant (p < 0.05) difference from cell type control. A cross (†) indicates a significant (p < 0.05) difference from concentration-matched WT cells.

Figure 2. MT related gene expression after arsenic exposure

MT-null or WT cells were exposed to various concentrations of arsenic for 24 h and expression of various MT related genes was assessed. A) *MT-I* transcript in cells treated with arsenic (note: there was no signal in MT-null cells); B) *MT-II* transcript in cells treated with arsenic (note: there was no signal in MT-null cells); C) *MT-I/II* protein in cells treated with arsenic (As; note: insert of blot indicating no signal in MT-null cells); D) *Mtf-1* transcript in cells treated with arsenic. Values (excluding the blot which is a representative experiment) represent the mean $\pm$ SEM (n = 4). An asterisk (*) indicates a significant (p

0.05) difference from cell specific control. A cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched MT-null cells.

Figure 3. Expression of *Abcc1* and *Abcc2* after arsenic exposure

MT-null or WT cells were exposed to various concentrations of arsenic for 24 h and *Abcc1* and *Abcc2* transcript levels were measured. A) *Abcc1* transcript in cells treated with arsenic; B) *Abcc2* transcript in cells treated with arsenic. Values represent the mean $\pm$ SEM ($n = 4$). An asterisk (*) indicates a significant ($p < 0.05$) difference from cell specific control. A cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched MT-null cells.

Figure 4. Expression of oxidant stress related genes after arsenic exposure

MT-null or WT cells were exposed to various concentrations of arsenic for 24 h and levels of *HO-1* and *GSTα2* were measured. A) *HO-1* transcript. B) *GSTα2* transcript. Values represent the mean $\pm$ SEM (n = 4). An asterisk (*) indicates a significant (p < 0.05) difference from cell specific control. A cross (†) indicates a significant (p < 0.05) difference from concentration-matched MT-null cells.

Figure 5. MT transfection into MT-null cells blocks arsenic cytolethality and ODD

MT-null cells were first transfected with the MT-I gene, then MT-null, WT or MT-null transfected with MT-I cells were exposed to arsenic for 24 h. A) Basal MT protein levels; B) *MT-I* mRNA levels in MT-null transfected with MT-I cells with arsenic exposure; C) WT, MT-null, or MT-null transfected with MT-I cells were exposed to various concentrations of arsenic for 24 h and cytolethality was assessed; D) ODD in WT, MT-null, or MT-null transfected with MT-I cells. Values represent the mean $\pm$ SEM (n = 4). An asterisk (*) indicates a significant (p < 0.05) difference from WT or cell specific control. A cross (†)

indicates a significant ($p < 0.05$) difference from MT-null cells treated in the same fashion. Western plot data are available from prior work for basal levels of MT protein in WT, MT-Null and MT-Null transfected with MT-I cells (Zuo et al., 2009).