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Effects of exogenous cysteine on inorganic and organic arsenicals-induced cytolethality†

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Inorganic arsenicals are distinctly toxic and carcinogenic to humans. Inorganic arsenite (As^{III}) and arsenate (As^{V}) are enzymatically methylated to monomethylarsonic acid ($MMAs^{V}$) and dimethylarsinic acid ($DMAs^{V}$) in mammals. Recent reports indicate that cytotoxic trivalent methylated arsenicals are produced through methylation of inorganic arsenicals and are involved in arsenic poisoning. Some previous studies have suggested that a typical thiol reagent cysteine (Cys) can reduce pentavalent arsenicals to trivalent arsenicals and might be able to enhance arsenic cytolethality. However, not much is known concerning the effects of exogenous Cys on the cytolethality of arsenicals. In this study, we examined the effects of exogenous Cys on the cytolethality induced by inorganic and organic arsenicals using rat liver cells. Cys prevented inorganic As^{III}-induced cytolethality. In contrast, when more than 5 mM Cys was incubated with millimolar levels of $MMAs^{V}$ or $DMAs^{V}$, $MMAs^{V}$- and $DMAs^{V}$-induced cytolethality significantly increased; this cytolethality might have been caused by the generation of trivalent methylated arsenicals. However, Cys at a concentration of less than 5 mM decreased the $MMAs^{V}$- and $DMAs^{V}$-induced cytolethality. These findings suggest that high concentrations of both arsenicals and Cys are required to form trivalent methylated arsenicals and to induce significant cytolethality. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; cysteine; dimethylarsinic; monomethylarsonic; Cys; Cys conjugate

INTRODUCTION

Arsenic is a metalloid element that is widely distributed in the environment as inorganic trivalent (arsenite; As^{III}) or pentavalent (arsenate; As^{V}) forms,[1] and its toxicity has been known since ancient times. In Asia and the Americas, chronic arsenic poisoning has occurred as a result of the consumption of water from wells drilled into arsenic-rich strata. Epidemiological studies have provided clear evidence that inorganic arsenicals are human carcinogens with target sites including liver, skin, lung, kidney and urinary bladder.[2] On the other hand, inorganic As^{III} has emerged as a potent chemotherapeutic agent with remarkable efficacy for certain human cancers such as acute promyelocytic leukemia.[3,4] It would thus appear that environmental and iatrogenic exposure to arsenicals will continue to be common.

In humans and numerous experimental animals, inorganic arsenicals are rapidly absorbed from the gastrointestinal tract and reduced to As^{III}.[5,6] Subsequently, it may be enzymatically methylated in the liver or other organs into organic arsenicals such as monomethylarsonic acid ($MMAs^{V}$) and dimethylarsinic acid ($DMAs^{V}$).[7] $MMAs^{V}$ and $DMAs^{V}$ are the major organic pentavalent arsenic metabolites in human urine after exposure to inorganic arsenicals.[5,7] It is believed that methylation of inorganic arsenicals results in a reduction in general toxicity, as indicated by their increased *in vivo* lethal dose in 50% of a population (LD_{50}) and *in vitro* lethal concentration in 50% of a

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population (LC_{50}).[8,9] However, the role of the methylation of inorganic arsenicals is not entirely clear and recent studies have increasingly suggested that the methylation of inorganic arsenicals is not a universal detoxification mechanism. It has been reported that trivalent methylated arsenicals such as monomethylarsonous acid ($MMAs^{III}$) and dimethylarsinous acid ($DMAs^{III}$) are found in urine collected from individuals who have been exposed to high concentrations of inorganic arsenicals;[10,11] further, synthetic trivalent methylated arsenicals such as monomethylarsine oxide ($MMAs^{III}O$) and iododimethylarsine ($DMAs^{III}I$) were more cytotoxic *in vitro* than inorganic and pentavalent methylated arsenicals.[12] It is generally believed that arsenicals react with various thiol reagents, and a typical thiol reagent, cysteine (Cys), might reduce pentavalent methylated arsenicals to trivalent methylated arsenicals in human body. Some studies have reported that Cys enhance arsenic cytolethality,[13–15] however, not much is known concerning the effects of exogenous Cys on the cytolethality of arsenicals.

In the present study, we observed the true effects of exogenous Cys on inorganic and organic arsenicals-induced cytolethality. This study may provide important information on the reaction between various arsenicals and thiol reagents.

EXPERIMENTAL

Chemicals

Sodium arsenite (As^{III}), sodium arsenate (As^{V}), and $DMAs^{V}$ were purchased from Wako Pure Chemical Co. (Osaka, Japan). $MMAs^{V}$ was purchased from Trichemical Co. (Yamanashi, Japan). These arsenicals were recrystallized twice, and their purities were >99.9% as determined by gas chromatography–mass spectrometry.[8,9] Endotoxin contamination of these arsenicals was not detected (<0.0000003%, wt/wt) using the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). L-Buthionin-(*S*,*R*)-sulfoximine [BSO; an inhibitor of γ-glutamylcysteine synthetase which decreases cellular reduced glutathione (GSH) levels], L-cysteine (Cys) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). L(−)-Cystine (Cys–Cys) was purchased from Wako Pure Chemical Co. (Osaka, Japan).

Cell culture

The TRL 1215 cells are nontumorigenic adhesive rat epithelial liver cells originally derived from the liver of 10-day old Fisher F344 rats.[16] TRL 1215 cells were cultured in William's E medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2:95% air at 37 °C.

Assay for cytolethality

Cells were isolated by trypsinization, washed twice and resuspended in fresh medium. 2×10^4 cells/100 μl/well were plated on flat-bottomed 96-well tissue culture plates and allowed to adhere to the plate for 24 h at 37 °C, at which time the medium was removed and replaced with 100 μl/well fresh medium containing the various test samples. Cells were then incubated with test samples for an additional 48 h at 37 °C. After incubation, cells were washed twice with warmed phosphate-buffered saline (pH 7.4) to remove non-adherent dead cells, and cell viability was determined by the AlamarBlue assay, which is similar to microtiter-tetrazolium (MTT) assay and measures metabolic integrity.[8,9] Briefly, after incubations with test samples and replacement with 100 μl/well fresh media, 10 μl/well AlamarBlue solution (Iwaki Grass Co., Chiba, Japan) was added directly to the 96-well plates, incubated for 4 h at 37 °C, and the absorbance at 570 nm (referenced to 600 nm) was measured by a microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA, USA). Data were expressed as metabolic integrity using the values from control cells, which were incubated with medium alone for 48 h at 37 °C, as 100%.

Thin layer chromatography (TLC)

TLC was performed with high performance TLC (HPTLC) plates silica gel 60 F 254 (Merck, Darmstadt, Germany) with a developing solvent of ethyl acetate : acetic acid : water (3 : 2 : 1). Iodide vapor was used for the detection of the various arsenicals.[8,17]

MTS assay

MTS assay was performed using Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega Co., Madison, WI).[18,19] This assay measures the amount of formazan produced by the metabolic conversion of Owen's reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] by Cys. The MTS is the subject of U.S. patent no. 5185450 and is licensed to Promega Co. Briefly, 15 μl of various concentrations of arsenicals were incubated with 15 μl of 1 mM Cys, 20 μl the kit's MTS solution, and 100 μl phosphate-buffered saline (pH 7.4) in 96-well microtiter plate at 37 °C during 120 min. The absorbance at 490 nm of formazan was measured by a microplate reader.

Statistical analysis

The data represent the mean plus or minus the standard error of the mean (SEM) and statistical evaluations were performed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test as appropriate.[20] A value of $p < 0.05$ was considered significant in all cases.

RESULTS

Effect of exogenous L-cysteine (Cys) on the cytolethality of As^{III}, As^{V}, $MMAs^{V}$ or $DMAs^{V}$

The effect of exogenous Cys on the cytolethality of As^{III}, As^{V}, $MMAs^{V}$ or $DMAs^{V}$ *in vitro* was examined. TRL 1215

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Table 1. Effect of exogenous Cys on arsenicals-induced cytolethality

Cys		−	+	−	+
BSO		−	−	+	+
Medium		100.0 ± 6.5	80.4 ± 3.6	103.3 ± 5.5	80.8 ± 1.3
As^{III}	10 μM	83.8 ± 4.5	82.7 ± 0.6	12.7 ± 4.3[a]	15.5 ± 7.8[a]
	25 μM	8.5 ± 0.6	63.6 ± 2.7[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
As^{V}	25 μM	99.0 ± 1.8	97.8 ± 1.1	3.2 ± 0.6[a]	0.0 ± 0.0[a]
	500 μM	15.8 ± 1.7	14.0 ± 0.7	3.6 ± 1.9	4.7 ± 3.4
$MMAs^{V}$	2.5 mM	93.8 ± 1.0	94.4 ± 2.1	94.6 ± 5.6	48.5 ± 5.5[a,c]
	10 mM	34.1 ± 0.2	31.1 ± 2.5	0.7 ± 0.0[a]	0.0 ± 0.0[a]
$DMAs^{V}$	5 mM	50.8 ± 2.5	75.3 ± 1.3[b]	97.3 ± 2.9[a]	93.9 ± 1.3[a]
	10 mM	6.0 ± 1.8	0.5 ± 0.5	78.8 ± 2.7[a]	0.4 ± 0.4[c]

TRL 1215 cells were preincubated with or without 50 μM BSO for 24 h. These cells were further exposed to As^{III} (10 or 25 μM), As^{V} (25 or 500 μM), $MMAs^{V}$ (2.5 or 10 mM), or $DMAs^{V}$ (5 or 10 mM) with or without 10 mM Cys in the presence or absence of 50 μM BSO for 48 h. Cellular viability was then assessed by the AlamarBlue assay. Results were expressed as metabolic integrity using the values from control cells, which were incubated with medium alone for 48 h at 37 °C, as 100%. Arithmetic mean plus/minus SEM of three separate experiments performed in triplicate, $n = 9$.
[a] $p < 0.001$, in comparison to the cells exposed to same concentrations of As^{III}, As^{V}, $MMAs^{V}$, or $DMAs^{V}$ alone,
[b] $p < 0.01$, [c] $p < 0.001$, in comparison to the cells simultaneously treated to both As^{III}, As^{V}, $MMAs^{V}$ or $DMAs^{V}$ and BSO.

cells were preincubated with or without 50 μM BSO for 24 h. These cells were subsequently exposed to As^{III}, As^{V}, $MMAs^{V}$ or $DMAs^{V}$ with or without 10 mM Cys in the presence or absence of BSO for 48 h, and then the cellular viability was assessed. We previously reported that As^{III}, As^{V} and $DMAs^{V}$ showed significant cytolethality in TRL 1215 cells; their LC_{50} values after 48 h exposure were 20 μM, 150 μM and 4.8 mM, respectively, and that $MMAs^{V}$ was not cytotoxic even at concentrations over 5 mM.[8,17–19,21–23] Exogenous Cys alone showed weak cytolethality in TRL 1215 cells at over 5 mM; the LC_{50} for Cys after 48 h exposure was 12.1 or 12.5 mM in the presence or absence of BSO pretreatment, respectively. Exogenous Cys did not augment cellular GSH levels in TRL 1215 cells during this incubation period (data not shown). BSO (50 μM) alone did not influence cell viability.

As shown in Table 1, exogenous Cys at a concentration of 10 mM significantly decreased the cytolethality of 25 μM As^{III}, although a decrease in the cytolethality was not observed in cellular GSH-depleted cells. The cytolethality of As^{V} was not affected by 10 mM Cys. The addition of 10 mM Cys strongly enhanced the cytolethality of 2.5 mM $MMAs^{V}$ in cellular GSH-depleted cells. The cytolethality of 5 mM $DMAs^{V}$ was significantly decreased by the addition of 10 mM Cys in the absence of 50 μM BSO. The addition of 10 mM Cys enhanced the cytolethality of 10 mM $DMAs^{V}$ in cellular GSH-depleted cells.

TRL 1215 cells were preincubated with or without 50 μM BSO for 24 h, and these cells were further exposed to 10 mM $MMAs^{V}$ or $DMAs^{V}$ with or without various concentrations of Cys in the presence or absence of 50 μM BSO for 48 h. The addition of <1.25 mM Cys did not affect $MMAs^{V}$-induced cytolethality; however, the addition of ≥1.25 mM Cys strongly prevented $MMAs^{V}$-induced cytolethality in the absence of BSO [Fig. 1(A)]. The addition of 1.25–5 mM Cys prevented $DMAs^{V}$-induced cytolethality in the absence of BSO; however, the addition of ≥5 mM Cys enhanced the cytolethality in cellular GSH-depleted cells [Fig. 1(B)].

The same results were obtained when the cells were simultaneously exposed to both arsenicals and *N*-acetyl-L-cysteine (NAC), which is similar in structure to Cys (data not shown).

Chemical reactions of As^{III}, As^{V}, $MMAs^{V}$ and $DMAs^{V}$ with Cys *in vitro*

We determined the plausibility of chemical reactions occurring between arsenicals and Cys. For this, we used MTS assays that indirectly demonstrated such reactions by measuring the competitive inhibitory effects of arsenicals on the reaction between Cys and MTS.[18,19] As shown in Fig. 2, while As^{III} strongly enhanced the Cys–MTS reaction in a dose-dependent manner, As^{V} enhanced this reaction to a slight extent. However, $MMAs^{V}$ and $DMAs^{V}$ significantly inhibited the Cys–MTS reaction in a dose-dependent manner. MTS did not react with any of the arsenicals or Cys–Cys alone (data not shown).

$MMAs^{V}$ and $DMAs^{V}$ easily combine with Cys in water

In order to determine the likelihood of the MMAs–Cys conjugate production, 1 mM $MMAs^{V}$ was incubated with or without 1, 4, 5 or 10 mM Cys in distilled water for 1 h at 37 °C. After the incubation, these mixtures were applied to an HPTLC plate and separated using ethyl acetate : acetic acid : water (3 : 2 : 1). Separated compounds were detected with iodide vapor. The TLC method have been authorized for separating and detecting of arsenic compounds.[8,17,24] As shown in Fig. 3(A), Cys [lane 1; relative mobility (Rf) = 0.45] and Cys–Cys (lane 2; $Rf = 0.11$) spots were detected with iodide vapor, but $MMAs^{V}$ was not detected under these experimental conditions (lane 3). The Cys spot did not appear

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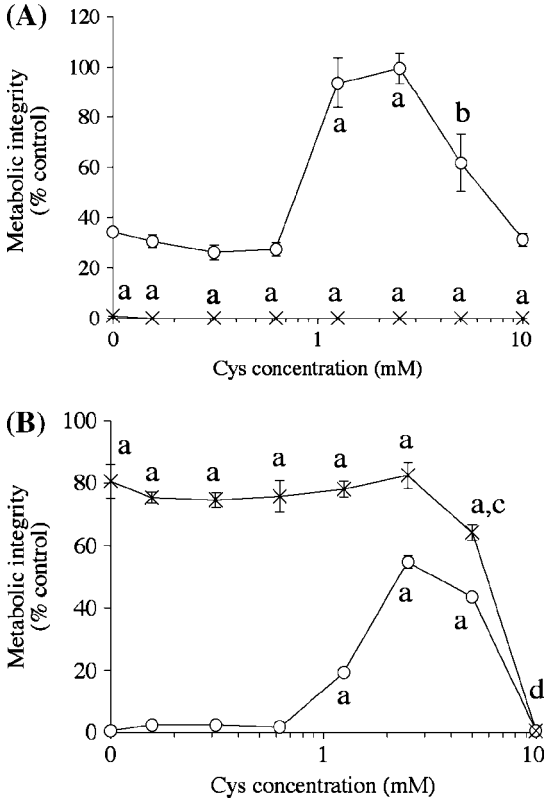

Figure 1. Effect of exogenous Cys on the cytolethality of $MMAs^V$ or $DMAs^V$. TRL 1215 cells were preincubated with (×) or without (○) 50 μM BSO for 24 h at 37 °C. After the preincubation, these cells were exposed to 10 mM $MMAs^V$ (A) or $DMAs^V$ (B) with or without various concentrations of Cys in the presence (×) or absence (○) of 50 μM BSO for 48 h at 37 °C. Cellular viability was then assessed by the AlamarBlue assay. Results are expressed as metabolic integrity using the values from control cells, which were incubated with medium alone for 48 h at 37 °C, as 100%. Arithmetic mean plus/minus SEM of three separate experiments performed in triplicate, $n = 9$. [a]$p < 0.001$, in comparison to the cells exposed to $MMAs^V$ or $DMAs^V$ alone, [b]$p < 0.01$; [c]$p < 0.01$, in comparison to the cells simultaneously treated to both $MMAs^V$ or $DMAs^V$ and BSO, [d]$p < 0.001$.

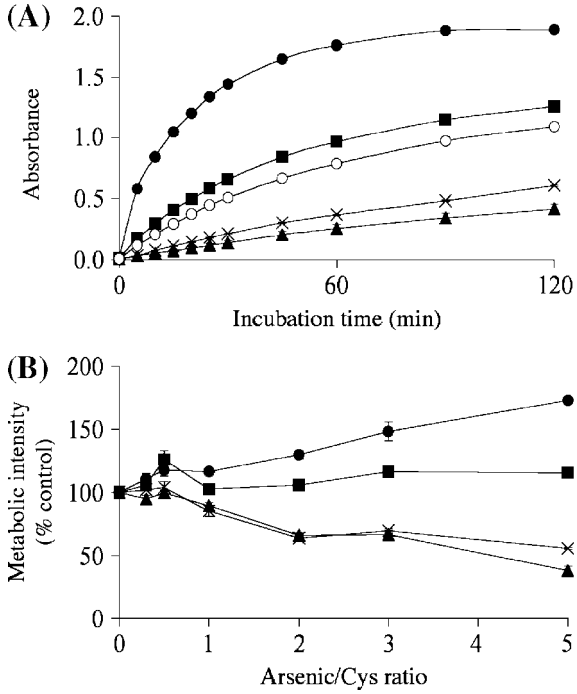

Figure 2. Inhibitory effect of arsenicals on the Cys-MTS reaction *in vitro*. (A) Cys was incubated with MTS in phosphate-buffered saline (pH 7.4) in the presence or absence (○) of As^{III} (●), As^V (■), $MMAs^V$ (▲) or $DMAs^V$ (×) *in vitro* at a concentration ratio of arsenical : GSH = 5 : 1, and the absorbance at 490 nm was measured during 120 min at 37 °C. (B) Cys was incubated with MTS in the presence or absence of various concentrations of As^{III} (●), As^V (■), $MMAs^V$ (▲), or $DMAs^V$ (×) *in vitro* for 120 min at 37 °C, and the absorbance at 490 nm was measured. One representative experiment out of three similarly performed is given. Data are expressed as raw absorbance (A) or metabolic integrity using the values from the control which was incubated with only Cys and MTS as 100% (B). Results are expressed as arithmetic mean plus/minus SEM ($n = 3$).

when 1 mM Cys was incubated with 1 mM $MMAs^V$ (lane 4), and a putative MMAs–Cys conjugate spot was detected at a different position from the Cys and Cys–Cys spots (lanes 5–7; $Rf = 0.18$) after incubating 1 mM $MMAs^V$ with ≥4 mM Cys.

We also examined the production of the DMAs–Cys conjugate from $DMAs^V$ and Cys using the same HPTLC system; 1 mM $DMAs^V$ was incubated with or without 1, 3, 5 or 10 mM Cys in distilled water for 1 h at 37 °C, and these mixtures were then applied to an HPTLC plate and separated. As shown in Fig. 3(B), $DMAs^V$ was not detected under these experimental conditions (lane 3). When 1 mM Cys was incubated with 1 mM $DMAs^V$, the Cys spot disappeared (lane 4). A putative DMAs–Cys conjugate spot (lanes 5–7; $Rf = 0.66$) was detected after incubating 1 mM $DMAs^V$ with ≥3 mM Cys.

In our experimental conditions, As^{III} was detected with iodide vapor on HPTLC plate, but As^V could not be detected. The spot density of As^{III} separated on HPTLC plate was not changed by the preincubation in distilled water for 48 h at 37 °C before spotting; this means that As^{III} was not oxidized to As^V in aqueous solution during 48 h incubation (data not shown).

Effect of the preincubation of $MMAs^V$ or $DMAs^V$ with Cys on their cytolethality

Low (1–100 μM) or high (40 mM) concentrations of $MMAs^V$ or $DMAs^V$ were preincubated with Cys at molar ratios of $MMAs^V$: Cys = 1 : 4 or $DMAs^V$: Cys = 1 : 3 in distilled water for 1 h at 37 °C. TRL 1215 cells were exposed to these

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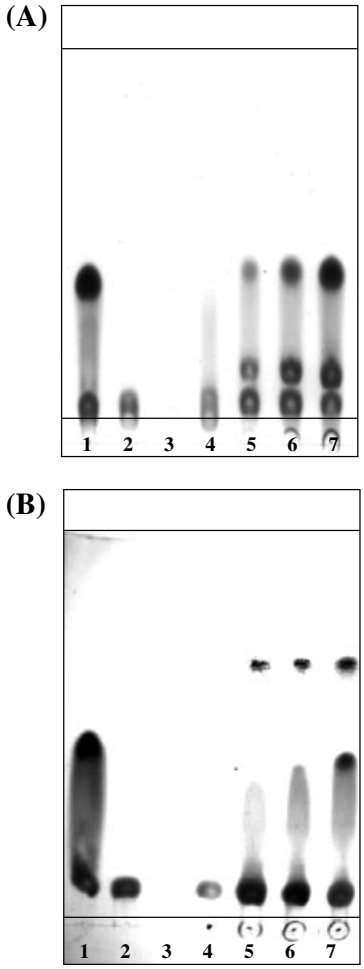

Figure 3. $MMAs^V$ and $DMAs^V$ easily combine with Cys in water. (A) $MMAs^V$ (1 mM) was incubated with (1, 4, 5 or 10 mM) or without Cys in distilled water for 1 h at 37 °C. After the incubation, aliquots (25 μL) of these mixtures were spotted on HPTLC plate, developed with solvent of ethyl acetate : acetic acid : water (3 : 2 : 1), and the separated spots were detected with iodide vapor. Lane 1, Cys (10 mM) only; lane 2, Cys–Cys (10 mM) only; lane 3, $MMAs^V$ (1 mM) only; lane 4, $MMAs^V$ (1 mM) plus Cys (1 mM); lane 5, $MMAs^V$ (1 mM) plus Cys (4 mM); lane 6, $MMAs^V$ (1 mM) plus Cys (5 mM); lane 7, $MMAs^V$ (1 mM) plus Cys (10 mM). (B) $DMAs^V$ (1 mM) was incubated with (1, 3, 5 or 10 mM) or without Cys in distilled water for 1 h at 37 °C. After the incubation, aliquots (25 μL) of these mixtures were spotted on HPTLC plate, developed with solvent of ethyl acetate : acetic acid : water (3 : 2 : 1), and the separated spots were detected with iodide vapor. Lane 1, Cys (10 mM) only; lane 2, Cys–Cys (10 mM) only; lane 3, $DMAs^V$ (1 mM) only; lane 4, $DMAs^V$ (1 mM) plus Cys (1 mM); lane 5, $DMAs^V$ (1 mM) plus Cys (3 mM); lane 6, $DMAs^V$ (1 mM) plus Cys (5 mM); lane 7, $DMAs^V$ (1 mM) plus Cys (10 mM).

arsenical–Cys mixtures at final arsenic concentrations up to 100 μM for 48 h, and cellular viability was then assessed.

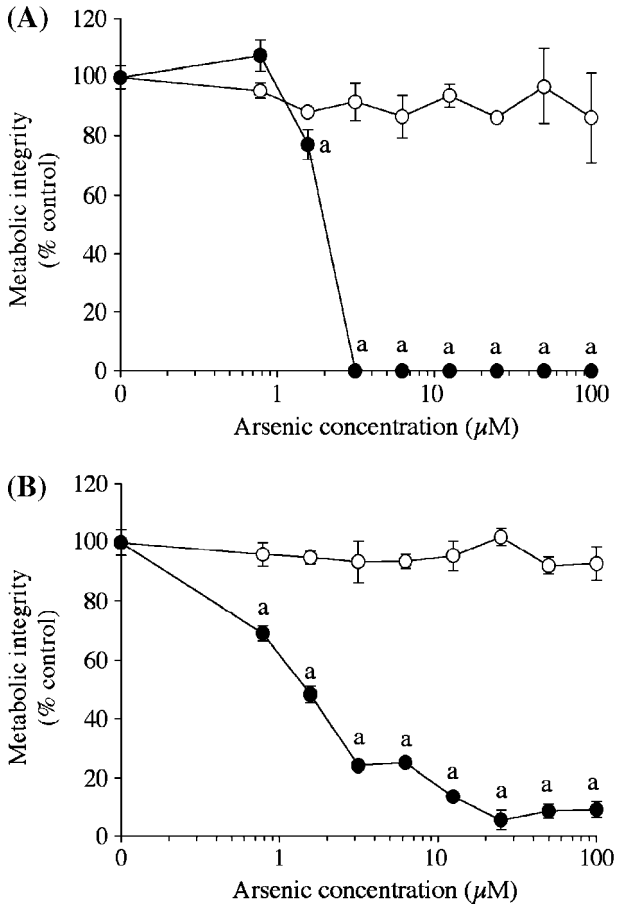

Figure 4. Effect of exogenous Cys on the cytolethality of $MMAs^V$ or $DMAs^V$; the mixtures of high-concentration $MMAs^V$ or $DMAs^V$ with Cys showed strong cytolethality. Low (1–100 μM; ○) or high (40 mM; ●) concentrations of $MMAs^V$ (A) or $DMAs^V$ (B) were preincubated with Cys at molar ratios of $MMAs^V$: Cys = 1 : 4 or $DMAs^V$: Cys = 1 : 3 in distilled water for 1 h at 37 °C. After the incubation, TRL 1215 cells were exposed to these arsenical–Cys mixtures at final arsenic concentrations up to 100 μM for 48 h at 37 °C. Cellular viability was then assessed by the AlamarBlue assay. Results are expressed as metabolic integrity using the values from control cells, which were incubated with medium alone for 48 h at 37 °C, as 100%. Arithmetic mean plus/minus SEM of three separate experiments performed in triplicate, $n = 9$. $^a p < 0.001$, in comparison to control cells.

As shown in Fig. 4, the mixture of low-concentrations of $MMAs^V$ or $DMAs^V$ with Cys was not cytotoxic. However, the mixture of high concentrations of $MMAs^V$ or $DMAs^V$ with Cys showed strong cytotoxicity (LC_{50} values as As; the mixture of 40 mM $MMAs^V$ with 160 mM Cys = 2.1 μM, the mixture of 40 mM $DMAs^V$ with 120 mM Cys = 1.3 μM). The same concentrations (up to 100 μM) of $MMAs^V$ or $DMAs^V$ alone were not cytotoxic.

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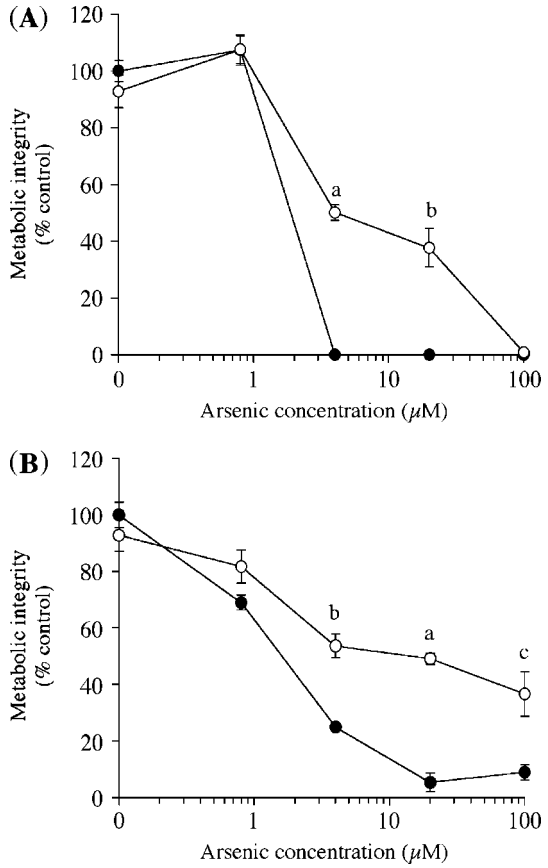

Figure 5. Effect of exogenous Cys on the cytolethality of the MMAs–Cys or DMAs–Cys mixtures. $MMAs^V$ or $DMAs^V$ at 40 mM was preincubated with 160 or 120 mM Cys, respectively, in distilled water for 1 h at 37 °C. TRL 1215 cells were exposed to these MMAs–Cys mixture (A) or DMAs–Cys mixture (B) at final arsenic concentrations up to 100 μM for 48 h at 37 °C in the presence (○) or absence (●) of 5 mM Cys. Cellular viability was then assessed by the AlamarBlue assay. Results are expressed as metabolic integrity using the values from control cells, which were incubated with medium alone for 48 h at 37 °C, as 100%; arithmetic mean plus/minus SEM of three separate experiments performed in triplicate, $n = 9$. $^a p < 0.001$, in comparison to cells exposed to the same concentrations of arsenicals in the absence of Cys. $^b p < 0.01$, $^c p < 0.05$.

Effect of exogenous Cys on the cytolethality of the MMAs–Cys or DMAs–Cys mixture

We also investigated the effects of exogenous Cys on the cytolethality of the MMAs–Cys or DMAs–Cys mixture. $MMAs^V$ or $DMAs^V$ at a high concentration, 40 mM, was preincubated with 160 or 120 mM Cys, respectively, for 1 h at 37 °C. TRL 1215 cells were exposed to various arsenic concentrations of these MMAs–Cys or DMAs–Cys mixtures for 48 h in the presence or absence of 5 mM Cys. It was found that exogenous Cys substantially reduced the cytolethality of the MMAs–Cys or DMAs–Cys mixture (Fig. 5).

DISCUSSION

Arsenic intoxication occurs in many areas through the consumption of contaminated well water or foods containing inorganic arsenicals.[2] Inorganic As^{III} has recently emerged as an outstanding chemotherapeutic agent with remarkable efficacy for certain human cancers such as acute promyelocytic leukemia.[3,4] In many mammalian species, inorganic As^V is first reduced to As^{III} and is subsequently methylated to $MMAs^V$ and $DMAs^V$.[5–7] The methylation of inorganic As^{III} and As^V was initially thought to be a detoxification process,[8,9] however, it was recently reported that toxic $MMAs^{III}$ and $DMAs^{III}$ might be produced through the methylation of inorganic arsenicals.[10–12,25–27] Scott *et al*. reported that a thiol reagent, reduced glutathione (GSH), reduced $MMAs^V$ and $DMAs^V$ to $MMAs^{III}$ and $DMAs^{III}$, resulting in the formation of monomethylarsonous diglutathione ($MMAs^{III}DG$) and dimethylarsinous glutathione ($DMAs^{III}G$).[28] We previously demonstrated that arsenicals and GSH concentrations greatly affected the formation and cytolethality of trivalent arsenicals.[17] In the human body, other thiol reagent such as Cys may also reduce pentavalent methylated arsenicals to trivalent methylated arsenicals. Some studies have suggested that Cys may enhance arsenic cytolethality;[13–15] however, not much is known concerning the effects of exogenous Cys on the cytolethality of arsenicals. Thus, studying the effects of Cys on the cytolethality of inorganic and organic arsenicals is important in order to define their *in vivo* cytolethality.

Cellular GSH depletion with BSO increased the cytolethality of As^{III}, As^V and $MMAs^V$, and reversely decreased the cytolethality of $DMAs^V$. We previously demonstrated that cellular GSH played an important role in the cytolethality of arsenicals; Cellular GSH prevented the cytolethality of As^{III}, As^V and $MMAs^V$, but was required for $DMAs^V$-induced cytolethality.[8,17–19,21–23] As^{III}-induced cytolethality was significantly decreased by the addition of Cys, however, this preventive effect of Cys disappeared when cellular GSH synthesis was stopped by BSO treatment. It has been reported that the treatment with Cys increase cellular GSH concentration when cellular GSH concentration is decreased, although Cys does not increase GSH concentration under normal, nonstressed, condition.[29,30] It has been reported that As^{III} consumes the cellular GSH when it shows cytolethality.[8,17–19,21–23] These results suggested that Cys decreased As^{III}-induced cytolethality by increasing cellular GSH content. As^{III} markedly enhanced the Cys-MTS reaction, and we previously reported that As^{III} also significantly enhanced the GSH-MTS reaction.[18,19] It has still not been clarified why As^{III} enhances these reactions; however, it is believed that this might, at least in part, be related to the mechanism of As^{III}-induced cytolethality or the protective effects of Cys against As^{III}-induced cytolethality. In contrast, Cys did not affect As^V-induced cytolethality. The effect of As^V on the reaction between Cys and MTS was very weak. In the previous study, the effect of As^V on the reaction between GSH and MTS was also found to be very weak.[18] These results

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indicate that As^V might weakly react with thiol reagents such as Cys or GSH. Further research is needed to verify the unknown role of nonenzymatic chemical reactions between inorganic arsenicals and thiol reagents on arsenical-induced cytolethality.

The addition of ≥5 mM Cys increased $MMAs^V$- and $DMAs^V$-induced cytolethality in cellular GSH-depleted cells. In this study, we demonstrated that $MMAs^V$ and $DMAs^V$ could easily combine with Cys *in vitro* using MTS assay and HPTLC system. These results indicated that exogenous Cys produced the cytotoxic arsenical–Cys conjugates. Scott *et al*. reported that $MMAs^V$ or $DMAs^V$ combined with GSH at molar ratios of $MMAs^V$:GSH = 1:4 or $DMAs^V$:GSH = 1:3 and formed $MMAs^{III}DG$ or $DMAs^{III}G$ *in vitro*.[28] We confirmed that the MMAs–Cys conjugate, which might be monomethylarsonous dicysteine ($MMAs^{III}DC$), was formed during the incubation of 1 mM $MMAs^V$ with ≥4 mM Cys and that the DMAs–Cys conjugate, which might be dimethylarsinous cysteine ($DMAs^{III}C$), was formed during the incubation of 1 mM $DMAs^V$ with ≥3 mM Cys. These results indicate that Cys reacts with $MMAs^V$ or $DMAs^V$ at the molar ratios of $MMAs^V$:Cys = 1:4 or $DMAs^V$:Cys = 1:3 and forms cytotoxic $MMAs^{III}DC$ or $DMAs^{III}C$ *in vitro*. When $MMAs^V$ or $DMAs^V$ was preincubated with Cys at molar ratios of $MMAs^V$:Cys = 1:4 or $DMAs^V$:Cys = 1:3, $MMAs^V$ or $DMAs^V$ was not cytotoxic when mixed in micromolar concentrations with Cys; however, when mixed in millimolar concentrations with Cys, $MMAs^V$ or $DMAs^V$ became strongly cytotoxic at the same final arsenic concentrations. These results suggest that the formation of cytotoxic $MMAs^{III}DC$ and $DMAs^{III}C$ depends on the arsenicals and Cys concentrations, and millimolar levels of both pentavalent methylated arsenicals and Cys are needed to form the arsenical–Cys conjugates that show significant cytolethality. It has been reported that arsenic concentrations in the plasma of chronic arsenic poisoning patients is at the micromolar level.[31] Therefore, it is not probable that large amounts of cytotoxic $MMAs^{III}DC$ and $DMAs^{III}C$ are produced nonenzymatically at *in vivo* levels of arsenicals and Cys.

Cys alone at concentrations of 5 mM or above showed significant cytolethality. Thus, the addition of very high concentrations, 10 mM, of $MMAs^V$ or $DMAs^V$ and ≥5 mM Cys might induce simple additive and/or synergistic cytotoxic effects of Cys, methylated arsenicals, and methylated arsenical–Cys conjugates.

On the contrary, the addition of <5 mM Cys with $MMAs^V$ or $DMAs^V$ decreased $MMAs^V$- and $DMAs^V$-induced cytolethality in the presence of cellular GSH. We confirmed that $MMAs^V$ and $DMAs^V$ could directly react with Cys *in vitro*. Additionally, exogenous Cys significantly decreased the cytolethality of the MMAs–Cys mixture and DMAs–Cys mixture. It was suggested that other synthetic trivalent methylated arsenicals, such as $MMAs^{III}O$ and $DMAs^{III}I$, became $MMAs^{III}(OH)_2$ and $DMAs^{III}OH$ in aqueous solution and showed significant cytolethality; their LC_{50} values were micromolar range.[32–34] We also recently demonstrated

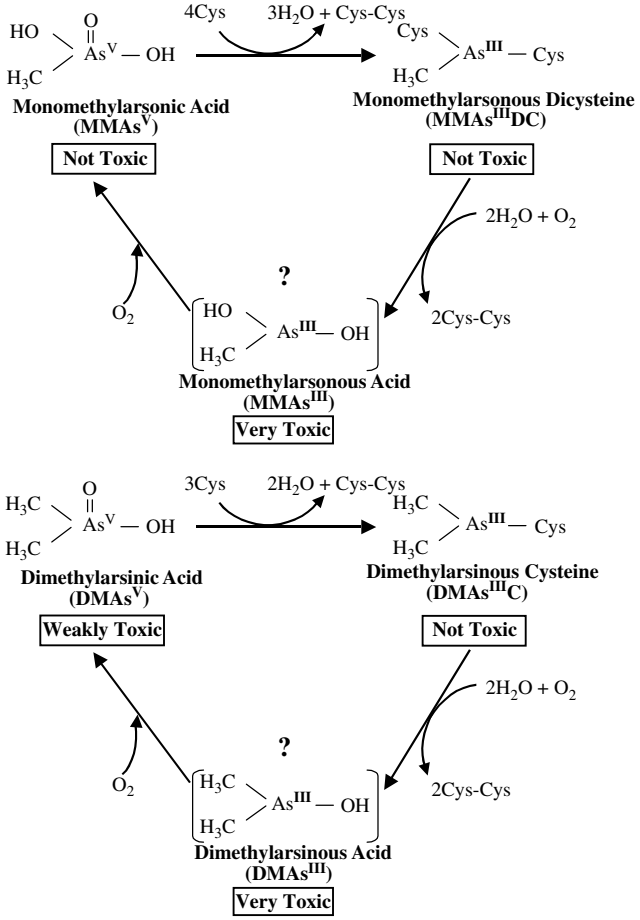

Figure 6. The putative nonenzymatic chemical reactions of monomethylarsenic or dimethylarsenic compounds with Cys.

that $MMAs^{III}DG$ or $DMAs^{III}G$, which was produced by incubation with $MMAs^V$ or $DMAs^V$ and GSH, might exert its cytolethality by separating into $MMAs^{III}$ or $DMAs^{III}$ and glutathione. These compounds are probably transformed into $MMAs^{III}(OH)_2$ or $DMAs^{III}OH$ and are transported into cells. Further, exogenous GSH maintains the form of $DMAs^{III}G$ and this conjugate cannot be transported into cells because the GSH molecule is not transported efficiently into cells.[17] Cys did not significantly augment cellular GSH concentration. It was reported that the Cys molecule was not transported efficiently into the cells under normal conditions.[29,30] Thus, it would be indicated that exogenous Cys less than 5 mM would partly produce the arsenical–Cys conjugates in the cell culture medium, prevented the cellular arsenic uptake, and then decreased $MMAs^V$- or $DMAs^V$-induced cytolethality. When the arsenical–Cys conjugates show significant cytolethality, they may separate into trivalent methylated arsenicals and Cys, probably becoming $MMAs^{III}(OH)_2$ and $DMAs^{III}OH$, before being transported into the cells (Fig. 6). Further examinations are needed to determine the chemical characteristics of the MMAs–Cys or DMAs–Cys conjugates in cell culture

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medium.

Thiol reagents, such as Cys and GSH, may be key molecules in preventing or inducing arsenic cytolethality. The present results suggested that arsenicals and Cys concentrations greatly affected the formation and cytolethality of these arsenical–Cys conjugates. Arsenicals and Cys concentrations higher than millimolar levels are needed to form the arsenical–Cys conjugates with significant cytolethality. The arsenical–Cys conjugates may exert their cytolethality by separating into trivalent methylated arsenicals and Cys, and being transported into the cells. *In vivo* levels (micromolar levels) of arsenicals and Cys may not nonenzymatically produce sufficient amounts of the cytotoxic arsenical–Cys conjugates. Therefore, the significant cytolethalities of $MMAs^{III}DC$ and $DMAs^{III}C$ may never manifest in the normal human body. Further research will be required in order to determine the role of Cys and methylation in the cytolethality of arsenicals in chronic arsenic poisoning patients who regularly ingest arsenic-contaminated well water and/or in acute promyelocytic leukemia patients who are injected with As^{III} as a chemotherapeutic agent.

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