— **Minireview** —

Biomethylation of Arsenic is Essentially Detoxicating Event

Teruaki Sakurai*

Laboratory of Environmental Chemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, Horinouchi 1432–1, Hachioji, Tokyo 192–0392, Japan

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Inorganic arsenic is clearly a toxicant and carcinogen in humans. In mammals, including humans, inorganic arsenic often undergo methylation, forming compounds such as pentavalent dimethyarsinic acid ($DMAs^V$). Recent evidence indicates that $DMAs^V$ is a complete carcinogen in rodents while evidence for inorganic arsenic as a carcinogen in rodents remains unclear. Thus, we studied the molecular mechanisms of the *in vitro* cytolethality of $DMAs^V$ compared to that of the trivalent inorganic arsenic, sodium arsenite, using rat liver TRL 1215 cells. Arsenite was very cytotoxic in these cells; its lethal concentration *in vitro* in 50% of a population (LC_{50}) was 20 μM after a 48-hr exposure. With arsenite, most dead cells showed histological and biochemical evidence of necrosis. The arsenite cytolethality markedly increased when cellular reduced glutathione (GSH) was depleted with the glutathione synthase inhibitor, L-buthionine-[*S*,*R*]-sulfoximine (BSO). In contrast, $DMAs^V$ was nearly three orders of magnitude less cytotoxic (LC_{50} = 1.5 mM) although evidence showed the predominating form of death was apoptosis. Surprisingly, GSH depletion actually decreased the $DMAs^V$-induced apoptosis. It is suggested that $DMAs^V$ requires intracellular GSH to induce apoptosis. Ethacrynic acid (EA), an inhibitor of glutathione *S*-transferase that catalyzes GSH-substrate conjugation, and aminooxyacetic acid (AOAA), an inhibitor of β-lyase which catalyzes the final breakdown of GSH-substrate conjugates, were also effective in suppressing the $DMAs^V$-induced apoptosis. These findings indicate that $DMAs^V$ was likely conjugated in some form with cellular GSH, and this conjugate induced apoptosis during subsequent metabolic reactions. Because apoptosis is a process by which organisms eliminate abnormal cells, the arsenic biomethylation in the human body may essentially a detoxicating event.

Key words —— arsenic, methylation, glutathione, dimethylarsinic acid, dimethylarsinous acid

INTRODUCTION

Arsenic is a chemical that evokes many images, most of which are negative. Inorganic arsenic is a common constituent of the earth's crust in its pentavalent form, and is widely distributed in soil and water. Humans may encounter inorganic arsenic in drinking water from wells drilled into arsenic-rich strata. Overt arsenic poisoning has occurred in some countries of Asia and the Americas through the consumption of contaminated well water or foods. Epidemiological studies have provided evidence that inorganic arsenic is a human carcinogen. Also, it is well known that inorganic arsenic has a strong acute toxicity in humans and experimental animals.[1)] An alternative approach to studies of the mechanism of arsenic carcinogenesis and toxicity has used cultured mammalian cells, and the findings obtained from cell culture studies have directly linked the exposure to inorganic arsenic to processes fundamental to carcinogenesis, however, there has been little information about the detailed evaluation and the mechanisms of the toxic effects of methylated arsenics which are metabolites of inorganic arsenic in mammals (Fig. 1). In humans and most experimental animals, pentavalent inorganic arsenate is rapidly reduced to trivalent arsenite, and then they are methylated as the principal metabolic reaction. *In vivo* studies suggest that methylated pentavalent arsenics, monomethylarsonic acid ($MMAs^V$) and dimethylarsinic acid ($DMAs^V$), have been identified as or-

*To whom correspondence should be addressed: Laboratory of Environmental Chemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, Horinouchi 1432–1, Hachioji, Tokyo 192–0392, Japan. Tel.: +81-426-76-6792; Fax: +81-426-76-5354; E-mail: sakurai@ls.toyaku.ac.jp

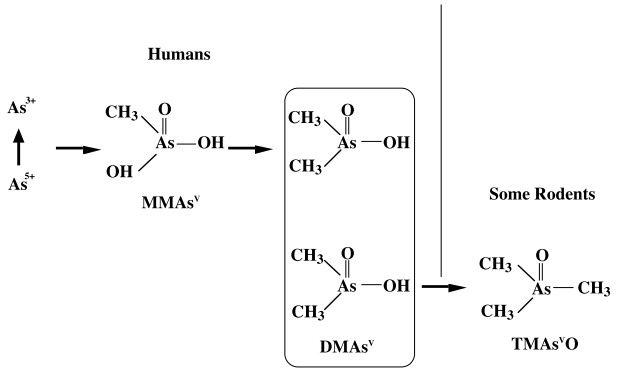

Fig. 1. The Arsenic Metabolism in Mammals

ganic metabolites in human urine after the administration of inorganic arsenic in either the trivalent or pentavalent state.[2,3] $DMAs^V$ is the ultimate metabolite in humans,[3] while the $DMAs^V$ is further methylated to pentavalent trimethylarsine oxide ($TMAs^VO$) in some rodents (Fig. 1).[2] It is believed that the methylation of inorganic arsenic results in the lowering of their general toxicity as indicated by their increased lethal dose *in vivo*[4] and *in vitro*,[5,6] but recent studies increasingly suggest that the methylation of inorganic arsenic is not a universal detoxification mechanism. It has been reported that $DMAs^V$ could significantly act as tumor promoters in rodents both *in vivo*[7] and *in vitro*,[8–10] however, further details on the toxic effects of $DMAs^V$ have not been reported. Thus, defining the mechanisms of chronic arsenic poisoning and carcinogenesis requires further studies on the toxicity of $DMAs^V$, the ultimate arsenic metabolite in humans, because inorganic arsenic is methylated and accumulated in arsenic poisoning patients.[11]

In this review, it is reported that our recent studies about the *in vitro* cytolethality of $DMAs^V$ compared with that of inorganic arsenite using TRL 1215 cells[12,13] which were derived from rat liver that is a major tissue for the metabolism of inorganic arsenic and is also a target tissue for their carcinogenic effects. We found that inorganic arsenite has a strong cytolethality; it induced necrosis in the cells at μM levels. In contrast, $DMAs^V$ was not cytotoxic at μM levels but it completely induced apoptosis at mM levels. Also, interestingly, $DMAs^V$ required intracellular reduced glutathione (GSH) to induce apoptosis, and this is quite different from inorganic arsenite and $MMAs^V$ for which GSH generally reduces the cytolethality. Apoptosis is a process by which organisms eliminate the abnormal cells, thus, the biomethylation of inorganic arsenic in the human body may essentially be a detoxicating event.

$DMAs^V$ Requires Cellular GSH to Induce Apoptosis[12]

The TRL 1215 cell line is a rat normally nontumorigenic epitherial liver cell line originally derived from the liver of 10-day old Fisher F344 rats.[14] Inorganic sodium arsenite was strongly cytotoxic in TRL 1215 cells; it's lethal concentration *in vitro* in 50% of a population (LC_{50}) after a 48-hr exposure was 20 μM (Fig. 2), and it primarily induced necrotic cell death indicated by the cellular morphological changes, nuclear terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and changes in the cell surface annexin-V expressions.[15] In contrast, $MMAs^V$ did not show any potent *in vitro* cytolethality even over 10 mM (Fig. 2). $DMAs^V$ showed weak but significant cytolethality in TRL 1215 cells; its LC_{50} was 1.5 mM which was much higher than that of inorganic arsenite (Fig. 2). Interestingly, $DMAs^V$ completely induced apoptosis at mM levels as indicated by the cellular morphological changes, condensed nuclei, nuclear TUNEL staining, changes in the cell surface annexin-V expressions and internucleosomal DNA fragmentation. We also previously reported similar results using mouse resident macrophages.[5] The reasons why $DMAs^V$ selectively induced apoptosis in mammal cells have not yet been precisely clarified. We demonstrated that intracellular GSH played key roles in inducing apoptosis by $DMAs^V$. In the case of inorganic arsenite, the depletion of cellular GSH with a GSH synthase inhibitor, L-buthionie-[*S*,*R*]-sulfoximine (BSO), significantly enhanced its cytolethality. $MMAs^V$ also became cytotoxic at mM levels when cellular GSH was depleted although it had no cytolethality in the presence of cellular GSH (Fig. 2). Cellular GSH must play an important role in protecting against the cytolethality of arsenite and $MMAs^V$ depending on both the chelation of these arsenic compounds and the scavenging of active oxygen produced by arsenite or $MMAs^V$ stimulation. This hypothesis was supported by results that the addition of exogenous GSH completely abolished the cytolethality of arsenite in TRL 1215 cells in the presence or absence of cellular GSH (Table 1). Conversely, BSO significantly prevented $DMAs^V$-induced apoptosis in a dose dependent manner (Fig. 2). The additions of other kinds of cellular GSH-deplet-

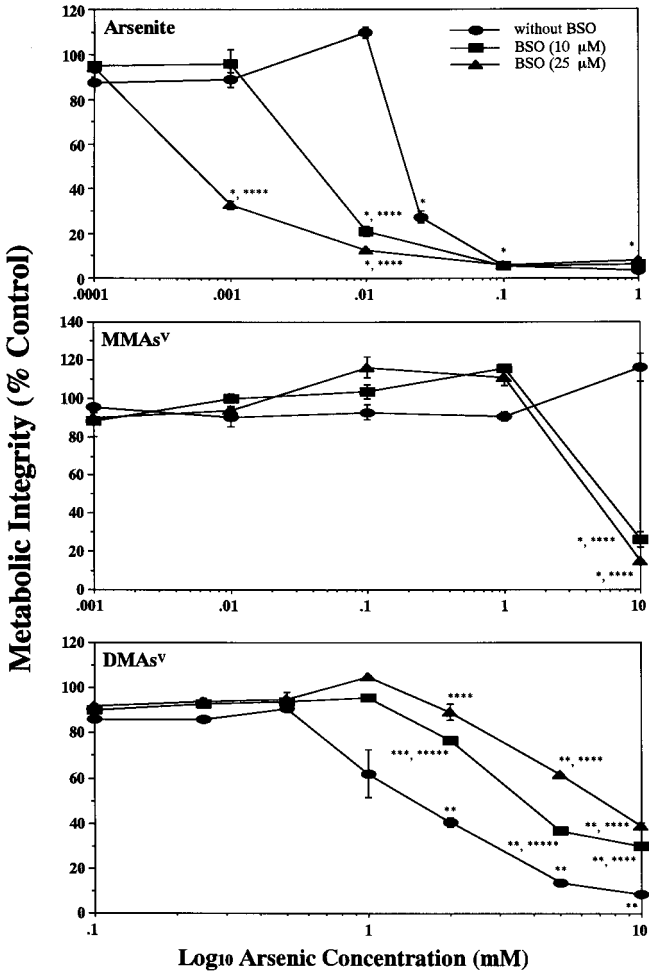

Fig. 2. Arsenic Cytolethality in TRL 1215 Cells

TRL 1215 cells were preincubated with 10 or 25 μM of BSO or without BSO for 24-hr, and continuously incubated with various doses of arsenite, $MMAs^V$ or $DMAs^V$ in the presence or absence of BSO for an additional 48-hr, and cell viability was measured. Results are expressed as mean ± SEM ($n = 3$). *$p < 0.001$, in comparison to cells incubated with medium alone by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. **$p < 0.01$. ***$p < 0.05$. ****$p < 0.001$, in comparison to cells incubated with the same arsenic but without BSO. *****$p < 0.01$, in comparison to cells incubated with the same arsenic and 25 μM BSO.

ing agents, such as diethyl maleate (a GSH scavenger) and carmustine (a glutathione reductase inhibitor), also significantly inhibited the $DMAs^V$-induced apoptosis in TRL 1215 cells. These findings suggest that $DMAs^V$ might selectively induce apoptosis that was related to the cellular GSH status. Styblo *et al.* and Zakharyan *et al.* previously described that cellular GSH was a critical cofactor for the promotion of the *in vitro* enzymatic methylation of arsenite and $MMAs^V$.[16,17] Thus, we tried to clarify if the ability to methylate arsenic was related to the role of cellular GSH in the $DMAs^V$ cytolethality using the chimpanzee skin CRL-1609 cell line because chimpanzee cells have no arsenic methyl transferase activities.[18] The treatment with BSO significantly decreased the cytolethality of $DMAs^V$ in the chimpanzee CRL-1609 cells like that used in the rat liver TRL 1215 cells that have potent arsenic methyl transferases (unpublished data). It is likely that the ability to methylate arsenic appears to have little to do with the role of GSH in the $DMAs^V$ toxicity.

In that study, we showed an interesting hypothesis that the conjugation of $DMAs^V$ and intracellular GSH might be an important event in the induction of apoptosis by $DMAs^V$ treatment (Fig. 3). When cellular GSH synthesis was stopped by BSO-treatment, the addition of $DMAs^V$ further decreased the cellular GSH levels compared with those of cells treated by BSO alone (unpublished data), and the BSO treatment slightly but significantly increased the cellular uptake of $DMAs^V$ in the TRL 1215 cells (Table 2). However, the addition of BSO actually

Table 1. Effect of BSO and/or Exogenous GSH on the Cytolethality of Arsenic in TRL 1215 Cells[a)]

arsenics	treatments	metabolic integrity (% control)
control	none	100.0 ± 1.2
	BSO	101.2 ± 1.0
	GSH	99.8 ± 1.5
	BSO + GSH	101.3 ± 1.6
arsenite	none	94.9 ± 0.6
	BSO	2.6 ± 1.5*,**
	GSH	88.5 ± 0.2*
	BSO + GSH	77.0 ± 1.0*,***,*****
$DMAs^V$	none	14.0 ± 0.5*
	BSO	26.2 ± 0.5*,**
	GSH	17.2 ± 0.7*,****
	BSO + GSH	30.9 ± 0.6*,**,*****

a) TRL 1215 cells were pre-incubated with or without BSO (25 μM) for 24-hr, and continuously incubated with arsenite (5 μM) or $DMAs^V$ (5 mM) in the absence or presence of BSO and/or exogenous GSH (250 μM) for an additional 48-hr, and the cell viability was measured. One representative experiment out of four similarly performed is given. Results are expressed as mean ± SEM ($n = 3$). *$p < 0.001$, in comparison to cells incubated with medium alone. **$p < 0.001$, in comparison to cells incubated with arsenic only. ***$p < 0.01$. ****$p < 0.05$. *****$p < 0.001$, in comparison to cells incubated with arsenic and BSO.

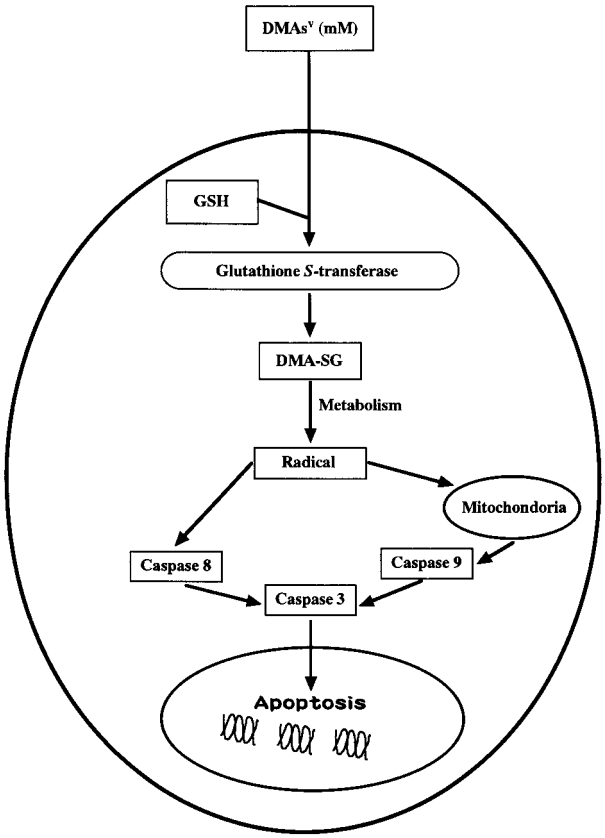

Fig. 3. The Putative Molecular Mechanism of DMA^V-Induced Apoptosis

Table 2. Effects of BSO and/or Exogenous GSH on the Cellular Uptake of DMA in TRL 1215 Cells[a)]

	As (mg/g cellular protein)
control	0.0 ± 0.0
$DMAs^V$	154.3 ± 2.2
$DMAs^V$ + BSO	216.9 ± 3.7*
$DMAs^V$ + GSH	160.7 ± 4.7
$DMAs^V$ + BSO + GSH	137.3 ± 2.2

a) TRL cells were pre-incubated with BSO (25 μM) for 24-hr, and continuously incubated with DMA (1 mM) for an additional 24-hr in the presence or absence of BSO and/or GSH (250 μM), and then, the amounts of arsenic contained in cell lysates were measured by atomic absorption spectophotpmetry. Results are expressed as mean ± SEM ($n = 3$). *$p < 0.001$, in comparison to cells incubated with $DMAs^V$ only.

increased cell survival after the $DMAs^V$ as described above. These data suggest that $DMAs^V$ by itself was not highly cytotoxic but it became cytotoxic and selectively induced apoptosis when it was conjugated with intracellular GSH (Fig. 3). This suggestion was supported by results that the addition of ethacrynic acid (EA), an inhibitor of glutathione *S*-transferase which catalyzes GSH-substrate conjugation, significantly inhibited the $DMAs^V$-induced apoptosis in TRL 1215 cells. We also demonstrated that the addition of acivicin, an inhibitor of γ-glutamyltranspeptidase which catalyzes the initial breakdown of the GSH-substrate conjugates, and aminooxyacetic acid (AOAA), an inhibitor of β-lyase which catalyzes the final breakdown of the GSH-substrate conjugates, significantly inhibited the $DMAs^V$-induced apoptosis. The combination of these GSH-substrate metabolic enzyme inhibitors and BSO showed a significant additive-like preventive effect on the $DMAs^V$-induced apoptosis. These data imply that $DMAs^V$ made a conjugate with the intracellular GSH, and this became cytotoxic during the subsequent metabolic reaction of the GSH-DMA conjugates in the cells (Fig. 3). In contrast, the treatment of EA significantly enhanced the cytolethality of inorganic arsenite in the TRL 1215 cells, although AOAA had no effect on it. Also, exogenous GSH completely prevented the arsenite-induced necrosis regardless of the presence of BSO as described above (Table 1). Therefore, the binding of GSH to arsenite might simply inhibit the cytolethality of arsenite. The addition of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), a potent cell membrane permeable radi-

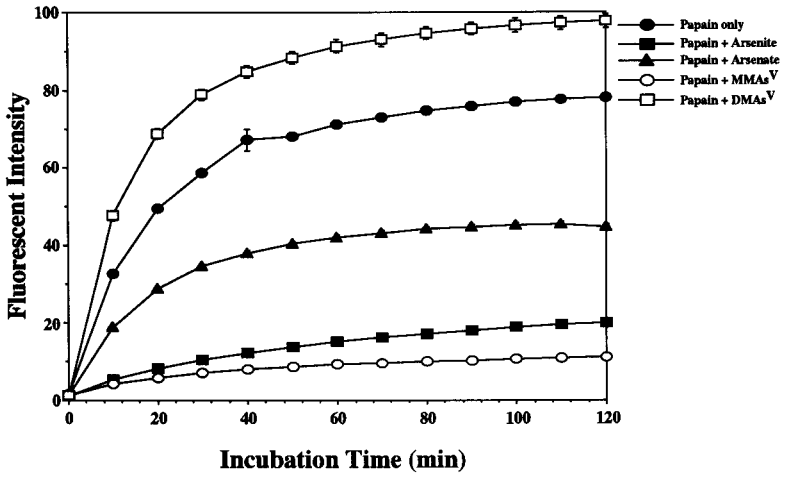

Fig. 4. Effects of Arsenic on the Cysteine Protease Activity *in Vitro*

Effects of arsenic on the cysteine protease activity were determined using EnzCheck™ Protease Kit (Molecular Probes, Co., Oregon, U.S.A.) and papain. 5×10^{-4} U/ml papain was incubated with 10 mg/ml fluorescein conjugated casein in the presence or absence of 200 mM arsenic during 120 min, and fluoresent intensity was measured. Results are expressed as mean ± SEM ($n = 3$).

cal trap reagent, strongly inhibited the $DMAs^V$-induced apoptosis in TRL 1215 cells, and a marked additive-like effect was observed upon inhibition of the $DMAs^V$-induced apoptosis by the combination of DMPO and BSO or inhibitors against the GSH-substrate metabolic enzymes. These data indicate that $DMAs^V$ caused the production of certain kinds of free radicals in the TRL 1215 cells, and these free radicals might play a key role in inducing the subsequent apoptosis (Fig. 3). Our preliminary results, using electron spin resonance spectroscopy and DMPO as a radical trapping reagent, indicated that an as yet unidentified radical species is formed by the $DMAs^V$ treatment in these cells, and current efforts are directed toward identification of this radical species. The most potent inhibitors of the $DMAs^V$-induced apoptosis did not affect the cellular uptake of the $DMAs^V$ in TRL 1215 cells. This result indicates that the significant preventive effects of these reagents on $DMAs^V$ cytolethality probably do not depend on the changes in the cellular uptake of the $DMAs^V$. Additionally, as shown in Fig. 4, we recently found that a unique biological effect of the $DMAs^V$; it directly stimulated the activity of a cysteine protease, papaine, *in vitro*, although other arsenics, including inorganic arsenite, arsenate and $MMAs^V$, strongly inhibited this enzyme activity. It is possible that the $DMAs^V$-induced apoptosis might be associated with the direct modulation of cysteine proteases because caspases known to regulate apoptosis are also cysteine proteases.

Thompson suggested in his previous review that GSH reduced $DMAs^V$ and that an unstable compound, such as dimethylarsinous acid ($DMAs^{III}$), might be formed,[19] and Scott *et al.* and Delnomdedieu *et al.* actually demonstrated that GSH reduced the $DMAs^V$ to $DMAs^{III}$ in water, resulting in the formation of the complex, $DMAs^{III}$-SG, using a nuclear magnetic resonance technique.[20,21] Recently, Styblo *et al.* reported that the *in vitro* cytolethality of $DMAs^{III}$-SG was much stronger than that of $DMAs^V$; its LC_{50} was similar with that of inorganic arsenite, 3–15 μM, using rat hepatocytes and human keratinocytes with chemically synthetic $DMAs^{III}$-SG.[1] Additionally, Le *et al.* and Mandal *et al.* reported that $DMAs^{III}$ were detected in human urine in the inorganic arsenic chronically affected areas in Inner Mongolia, China or West Bengal, India, respectively.[22,23] Thus they suggest that the biomethylation of arsenics in mammals was not always a detoxification mechanism. However, under our experimental conditions shown there, $DMAs^V$-induced apoptosis in TRL 1215 cells might not be mediated *via* the formation of a toxic GSH-DMA complex, such as $DMAs^{III}$-SG. The LC_{50} of $DMAs^V$ in TRL 1215 cells, 1.5 mM, was much higher than that of $DMAs^{III}$-SG which was estimated by Styblo *et al.*,[1] and $DMAs^{III}$-SG did not induce apoptosis under their experimental conditions.[1] We also observed that GSH actually could easily combine with $DMAs^V$ in phosphate buffered saline (PBS; pH = 7.4) using high performance thin layer chromatography. However, exogenous GSH (250 μM) slightly and significantly inhibited, but never enhanced, the

$DMAs^V$ cytolethality regardless of the presence of BSO in the TRL 1215 cells (Table 1). Also, as shown in Table 2, exogenous GSH (250 μM) did not affect at all the cellular uptake of $DMAs^V$. These data imply that exogenous μM levels of GSH might not be able to become cytotoxic together with $DMAs^V$ even though certain kinds of GSH-DMA complexes might be formed in the cell culture medium. Similar results were observed at every $DMAs^V$/GSH ratios when μM levels of GSH were used, and the addition of other thiol reductants, such as L-cysteine and *N*-acethyl-L-cysteine, also showed similar effects with GSH. Ochi *et al*. reported that toxic trivalent dimethylarsine gas might be formed in the reaction between $DMAs^V$ and GSH in a cell culture medium.[24] We also examined if trivalent dimethylarsine gas was generated from the DMA-GSH mixture. Actually, a smelly gas was generated when purchased $DMAs^V$ was mixed with the same concentration of GSH in PBS, but most of this gas was trivalent trimethylarsine determined by gas chromatography-mass spectrometry. Thus, we reexamined using twice recrystallized $DMAs^V$, and no gas was detected with GSH. GSH also did not generate any gas with $MMAs^V$, but it generated trivalent trimethylarsine gas with $TMAs^VO$. This strongly suggests that purchased $DMAs^V$ contains a small amount of $TMAs^VO$ as an impurity, and this impurity was reduced and converted to the smelly trimethylarsine gas with GSH. The degree of gasification of methyl arsenic compounds is dependent upon the number of methyls in the chemical structures. A higher reducing effect on methylated arsenics was observed from *N*-acethyl-L-cysteine than from GSH. Taken together, $DMAs^V$ may easily forms a soluble, non gas, complex with μM levels of GSH in the cell culture medium, but this complex may not be very cytotoxic. However, the cytolethality of mM levels of $DMAs^V$ was sometimes significantly enhanced by the addition of high concentrations, mM levels, of GSH at every $DMAs^V$/GSH ratios.[5,24] These findings indicate that high concentrations of $DMAs^V$ and/or GSH might be needed to make these chemicals react and to keep the toxic unsuitable chemical form of the GSH-DMA complex, such as $DMAs^{III}$-SG, in the cell culture medium because this complex might be rapidly oxidized in a normal cell culture medium, or it might be simple additive and/or synergistic cytotoxic effects between mM levels of $DMAs^V$ and GSH because GSH itself was also cytotoxic at mM levels like $DMAs^V$; its LC_{50} in TRL 1215 cells was 12 mM and it alone showed a significant cytolethality from 10 mM. Further examinations are needed to verify the chemical characteristics and cytolethality of this putative GSH-DMA complex in the cell culture medium, however, a toxic GSH-DMA complex, $DMAs^{III}$-SG, has not been detected yet in both human urine obtained from chronic arsenicosis patients[22,23] and tissue samples from experimental animals which were injected with arsenic,[25] although only soluble dimehylarsinous acid (DMA^{III}) was detected from these samples.[22,23,25]

Recent evidence suggests that soluble DMA^{III} was strongly cytotoxic like inorganic arsenite in mammalian cells *in vitro* using synthetic iododimethlarsine ($DMAs^{III}I$) as a source of this arsenic chemical,[1,26,27] and DMA^{III} was actually detected from the animal tissue samples after the arsenic injection as a temporary intermediate of *in vivo* arsenic biomethylation.[25] Thus, as described above, it is suggested that arsenic biomethylation is not always the detoxification mechanism. However, some mammalian species, such as the marmoset monkey and the chimpanzee, have only a low resistance to arsenic because they have been shown not to be able to methylate the inorganic arsenic at all,[28] and serious clinical conditions of arsenic poisoning patients were sometimes observed in children because they have a lower degree of methylation of arsenic than adults in humans.[28] Suzuki *et al*. demonstrated using rat models that all of the injected inorganic arsenic was finally metabolitde to $DMAs^V$, and excreted into the urine.[25] The *in vitro* cytolethality of $DMAs^V$ was much lower than that of inorganic arsenite as described in this review, and $DMAs^V$ selectively induced apoptosis *via* binding with intracellular GSH (Fig. 3). $DMAs^V$-induced apoptosis was observed in various species of mammalian cells, including human peripheral blood monocytes, human skin fibroblasts, chimpanzee skin fibroblasts, rat liver cells and mouse macrophages.[5,12,13] We also previously reported that $DMAs^V$ suppressed the release of inflammatory cytokines, such as tumor necrosis factorα and interleukin 1α, from mouse macrophages by induction of apoptosis, although inorganic arsenics induced a marked release of them in concert with necrosis.[5] Because apoptosis is a process by which organisms eliminate the damaged, precancerous or redundant cells without invoking an inflammatory responses, the biomethylation of inorganic arsenic in the human body may essentially be a detoxicating event by not only reducing its acute

toxicity but also reducing the inflammatory potency.

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