Current Topics

Advances in Biology and Toxicology of Environmental Metals/Metalloids

Role of Arsenic (+3 Oxidation State) Methyltransferase in Arsenic Metabolism and Toxicity

Daigo Sumi* and Seiichiro Himeno

Laboratory of Molecular Nutrition and Toxicology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University; Yamashiro-cho, Tokushima 770–8514, Japan.

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The metabolism of arsenicals, including their reduction and methylation has been extensively studied, and both classical and novel pathways of arsenic methylation are proposed. Arsenic methylation has been considered to be a detoxification process of inorganic arsenicals, although recent studies have indicated that trivalent methylated arsenicals, the intermediate products of arsenic methylation, are more toxic than inorganic arsenicals. In 2002, arsenite (+3 oxidation state) methyltransferase (As3MT) was discovered to be an enzyme responsible for arsenic methylation. This review focuses on current information on the function, genetic polymorphism, and alternative splicing of As3MT, all of which contribute to arsenic metabolism and toxicity.

Key words arsenic; methylation; arsenite (+3 oxidation state) methyltransferase; polymorphism; alternative splicing

1. INTRODUCTION

Arsenic is an environmental pollutant derived from the Earth's crust.¹⁾ Many people around the world are exposed to arsenic-polluted underground water from wells and suffer from chronic arsenic poisoning, which causes hyperkeratosis, cancer, diabetes, cardiovascular disease, and other diseases.^{2–6)} On the other hand, arsenic trioxide has been used to treat acute promyelocytic leukemia (APL), and is especially effective in APL patients who have acquired resistance to all-*trans* retinoic acid therapy.^{7–9)}

Since the mid-1970s, studies have extensively explored the metabolism of arsenicals, including the reduction of inorganic arsenate (As(V)) to arsenite (As(III)) and the following methylation of inorganic arsenicals to mono- (MMA) and di-methylated forms (DMA) that are excreted *via* urine.¹⁰⁾ The omega isoform of glutathione *S*-transferase (GSTO) and purine nucleoside phosphorylase (PNP) each catalyze the reduction of As(V) to As(III).^{11,12)} Recently, polynucleotide phosphorylase and mitochondrial ATP synthase were also reported to reduce As(V) to As(III) in the presence of glutathione (GSH).¹³⁾ The methylation of As(III) requires *S*-adenosylmethionine (SAM) as a methyl donor and produces methylated arsenicals including monomethylarsonate (MMA(V)), monomethylarsonous acid (MMA(III)), dimethylarsinic acid (DMA(V)), and dimethylarsinous acid (DMA(III)). In 2002, Thomas and colleagues identified arsenite (+3 oxidation state) methyltransferase (As3MT) from rat liver cytosol as an enzyme responsible for arsenic methylation.¹⁴⁾ Thus far, two pathways have been proposed for the reactions of arsenical methylation in mammalian systems. Aposhian and colleagues described a pathway, now called the classical pathway, in which inorganic arsenic is metabolized *via* oxidative methylation.^{15,16)} In the classical

pathway, it has been considered that As3MT catalyzes the oxidative methylation steps from As(III) to MMA(V) and from MMA(III) to DMA(V).^{14,17)} On the other hand, Hayakawa *et al.*¹⁸⁾ proposed a novel pathway in that GSH conjugation of As(III) occurs first and then the methylation of the GSH-conjugate of As(III) yields a monomethylarsonic diglutathione. In either pathway, arsenic methylation has been considered a detoxification process of inorganic arsenicals, although recent studies have indicated that MMA(III) and DMA(III), the intermediate products of arsenic methylation, are more toxic than inorganic arsenicals.^{19–21)}

Because the manifestation of arsenic toxicity depends on its accumulation in the organs and cells, the function and expression of As3MT that catalyzes arsenic methylation play central roles in arsenic metabolism and toxicity, as do the transporters that govern arsenic influx and efflux and the transcription factors such as nuclear factor (NF)-E2-related factor 2 (Nrf2) that activate cellular protection systems upon exposure to arsenicals.^{15,22)} However, there have been few review articles on the relationship between As3MT and arsenic metabolism and toxicity. This review focuses on the current information on the function, genetic polymorphism, and alternative splicing of As3MT, all of which contribute to arsenic metabolism and toxicity.

2. EFFECT OF ARTIFICIAL MANIPULATION OF AS3MT EXPRESSION

To understand the role of As3MT in arsenic methylation and detoxification, silencing or overexpression of the *As3MT* gene is a valuable measure. Drobná *et al.*²³⁾ examined the effect of short hairpin RNA (shRNA) silencing of As3MT on the methylation capacity of human hepatocarcinoma HepG2

* To whom correspondence should be addressed. e-mail: sdaigo@ph.bunri-u.ac.jp

cells after exposure to As(III). The results indicated that the introduction of As3MT shRNA decreased the arsenic methylation in HepG2 cells. However, the methylation capacity of the clonal cells stably transfected with As3MT shRNA decreased only to 30% that of the nontransfected cells, whereas shRNA transfection reduced the protein level of As3MT to 12% that of the nontransfected cells. This suggests that the As3MT-independent pathway may also exist in HepG2 cells and contribute partly to arsenic methylation. Recently, Ren *et al.*²⁴ identified N-6 adenine-specific DNA methyltransferase (N6AMT1) as a novel arsenic methyltransferase.

The metabolism and toxicity of As(III) and MMA(III) were examined in human bladder-derived UROtsa cells that lack endogenous As3MT expression. The transfection of rat *As3MT* by using a retroviral gene delivery system into UROtsa cells led to an effective formation of methylated arsenicals compared with parental UROtsa cells.²⁵ It was expected that the As3MT-transfected UROtsa cells were more resistant to the cytotoxicity of As(III) and MMA(III). However, these cells were more sensitive to the cytotoxicity of As(III) at high concentrations (50–100 μM) with a concomitant decrease in the formation of DMA from MMA. These data suggest that the concentrations of 50–100 μM As(III) inhibited DMA formation, resulting in an accumulation of intracellular MMA, probably present as MMA(III), which is a cytotoxic species. These data also suggest that a high concentration of As(III) may inhibit As3MT activity to a greater degree for the formation of DMA rather than that of MMA. Regarding this point, Song *et al.*²⁶ examined the enzymology of As3MT by using recombinant human As3MT. The results proved that DMA formation after As(III) incubation with As3MT increased with a peak at 8.3 μM of As(III), but then began to decrease, finally to the level of no activity. On the other hand, the formation of MMA increased with a peak at 100 μM and then decreased, suggesting that the inhibitory effect of As(III) on the conversion of MMA to DMA is greater than that of As(III) to MMA. However, the mechanisms underlying the inhibition of the conversion of MMA to DMA by arsenicals remain unclear.

In the case of the cytotoxicity of MMA(III), As3MT-transfected UROtsa cells were more resistant to MMA(III) at concentrations of 1–3 μM . In these cells, the predominant form of intracellular arsenic after incubation with MMA(III) is DMA. These results suggest that the resistance to MMA(III) in As3MT-transfected UROtsa cells was acquired by the ability to convert MMA to DMA. However, the resistance of As3MT-transfected UROtsa cells was weakened when the cells were exposed to 4–5 μM of MMA(III) with a concomitant decrease in DMA formation and an increase in MMA accumulation. These results suggest that a high concentration of MMA(III) may also inhibit the formation of DMA from MMA(III) in cells.

Drobna *et al.*²⁷ produced *As3MT* knockout mice to investigate the *in vivo* roles of As3MT in the distribution, metabolism, and excretion of arsenicals. The results showed that arsenic concentrations in organs such as liver, kidneys, and lungs were higher in *As3MT* knockout mice than in wild-type mice after a single oral dose of As(V). The whole body clearance of arsenic in *As3MT* knockout mice was considerably slower than that in wild-type mice. Recently, two studies reported the changes in the toxicity of As(III) by using *As3MT* knockout mice. In the first study, *As3MT* knockout mice

exhibited severe and lethal systemic toxicity during the first week of As(III) exposure from the diet (100, 150 ppm). The arsenic-containing granules in the superficial urothelial layer were more abundant and larger in *As3MT* knockout mice than in wild-type mice.²⁸ The second study showed that simple hyperplasia in the bladder was more pronounced in *As3MT* knockout mice than in wild-type mice after administration of 50 ppm As(III) from drinking water for 4 weeks.²⁹ Also, mild hepatitis in the liver and hydronephrosis in the kidney were observed in *As3MT* knockout mice, but not in wild-type mice, after administration of 25 ppm As(III) from drinking water for 4 weeks. These results suggest that As3MT plays an important role in protection against arsenic toxicity in various organs in mice, including liver, kidney, and bladder.

3. GENETIC POLYMORPHISM OF *AS3MT* GENE (FIG. 1)

The As3MT catalyzes the conversion of As(III) to MMA and of MMA to DMA, as mentioned above. Since it is difficult to determine the enzymatic activity of As3MT in human tissues, the profiles of urinary arsenic metabolites have been utilized as a surrogate indicator of an individual's arsenic methylation ability. The ratios of urinary concentrations of MMA/inorganic arsenic (As(III)+As(V)) and DMA/MMA have been used to show the ability to complete the first and second steps of methylation, respectively. Concha *et al.*³⁰ examined urinary arsenic metabolites in children and adult women in three villages in northern Argentina. The results showed that a higher percentage of inorganic arsenic and a lower percentage of DMA in total arsenic metabolites in urine were found in children than in adult women, suggesting that these children are more sensitive than adult women to the toxic effects of arsenic due to children's lower ability to methylate inorganic arsenicals. Some investigations have revealed that the higher percentage of MMA in urinary arsenic metabolites is related to adverse health effects.^{31–33} Del Razo *et al.*³⁴ examined the relationships between the profiles of urinary arsenic metabolites and the presence of cutaneous symptoms among individuals chronically exposed to arsenic in Mexico. The individuals with cutaneous symptoms had higher ratios of MMA/total arsenic metabolites and MMA/inorganic arsenic, and a lower ratio of DMA/MMA, than the individuals without cutaneous signs.

Hereditary factors are implicated in the individual variations of arsenic metabolism. The amounts of MMA in urine were shown to vary among populations exposed to arsenic via drinking water.³⁵ Examination of the primary human hepatocytes obtained from healthy donors revealed that there was up to a seven-fold difference in the methylation rate of As(III) among eight donors.³⁶ To understand the relationship between genetic variants of As3MT and arsenic metabolism, Meza *et al.* examined the associations of the urinary arsenic metabolites with 23 types of polymorphisms of the genes involved in arsenic metabolism such as *GSTO*, *PNP*, and *As3MT* among the arsenic-exposed subjects living in the Yaqui Valley of Sonora, Mexico.³⁷ The results showed that the ratio of urinary DMA/MMA in the children (7–11 years of age) were significantly associated with three intronic polymorphisms in *As3MT* (rs12767543, rs3740393, and rs11191453), but not with the polymorphisms in *GSTO* and *PNP* genes. Schläwicke

Fig. 1. Relationships between Individual Variations in Arsenic Metabolism and Toxicity with Genetic Polymorphisms in *As3MT* Gene

↑ shows increase and ↓ shows reduction.

Engström *et al.*³⁸⁾ tested the associations of the polymorphisms in the *As3MT* gene with urinary arsenic metabolites among women living in San Antonio de los Cobres, a village in the northern Argentinean Andes. They found significant associations of the three intronic polymorphisms in *As3MT* (rs3740393, rs3740390, and rs10748835) with the decrease in the percentage of MMA and the increase in the percentage of DMA in urinary arsenic metabolites, suggesting that these polymorphisms are associated with the second step of arsenic methylation.

In addition to intronic polymorphisms, an exonic polymorphism in the *As3MT* gene (rs11191439) has been well examined since this polymorphism results in the substitution of amino acid from Met to Thr at 287 in *As3MT* (Met287Thr). In order to examine the effect of this substitution on methyltransferase activity, cDNA encoding wild-type or Met287Thr *As3MT* was transfected into COS-1 cells.³⁹⁾ The methyltransferase activity in the cells transfected with Met287Thr *As3MT* was higher than that in the cells transfected with wild-type *As3MT*. However, it remains unknown why Met287Thr *As3MT* protein has higher methyltransferase activity than the wild-type protein. The association of Met287Thr substitution of *As3MT* with arsenic metabolism was also examined among various human populations. ASHRAM (Arsenic Health Risk Assessment and Molecular Epidemiology), a case-control study of cancer risks

in relation to low-level arsenic exposure *via* drinking water in central Europe,⁴⁰⁾ revealed that Met287Thr polymorphism is associated with a higher percentage of MMA and a lower percentage of DMA in urinary arsenic metabolites in the populations of Hungary, Romania, and Slovakia. Hernández *et al.*^{41,42)} reported that individuals with Met287Thr *As3MT* showed higher percentages of MMA in urinary arsenic metabolites than individuals with wild-type *As3MT* among Chilean men exposed to arsenic. In a study of residents of the Red River Delta, Vietnam, Agusa *et al.*⁴³⁾ also showed that individuals with heterozygous SNP for Met287Thr had higher percentages of MMA in urinary arsenic metabolites. The allele frequency of Met287Thr mutation varies among ethnic groups: Xhosa (0.233), Chilean (0.140), Central European (0.109), African (0.108), and Caucasian (0.100) populations had higher frequencies than in Asian populations such as Mongolian (0.040), Korean (0.010), and Japanese (0.010).^{39,41,44,45)}

Taken together, the results obtained from population studies suggest that Met287Thr substitution of *As3MT* results in an increase in the capacity of the first step of methylation, leading to an increase in the percentage of urinary MMA, but not the second step of methylation. Since an *in vitro* study clearly showed that Met287Thr *As3MT* protein has higher methyltransferase activity than the wild-type protein,³⁹⁾ further studies are necessary to elucidate the function of *As3MT* in the

Fig. 2. Alternative Splicing of *As3MT* mRNA

second step of arsenic methylation.

A recent study demonstrated that there is a positive association between DNA damage in the peripheral blood leukocytes and the frequency of the Met287Thr variant allele in children living in Torreon, Coahuila, Mexico. The finding that Met287Thr polymorphism affects the susceptibility to DNA damage suggests that a higher activity of *As3MT* might increase the risk of adverse health effects among populations chronically exposed to arsenic.⁴⁶⁾

Engström *et al.*⁴⁷⁾ examined the associations among mRNA expression of *As3MT*, arsenic metabolism, and eight polymorphisms of *As3MT* (intronic: rs708514, rs3740400, rs3740393, rs3740390, rs11191453, rs10748835, and rs1046778; exonic: rs11191439 (Met287Thr)) in populations living in Argentina (San Antonio de los Cobres, Tolar Grande, Olacapat, and Salar de Pocitos) and Bangladesh (Matlab, a rural area 53 km southeast of Dhaka). Three of the intronic polymorphisms (rs3740393, rs3740390, and rs11191453) showed higher percentages of inorganic arsenic and lower percentages of DMA in urinary arsenic metabolites in both populations. Three other intronic polymorphisms (rs3740400, rs10748835, and rs1046778) showed higher percentages of MMA in urinary arsenic metabolites in both populations. In addition, the exonic polymorphism (rs11191439) showed significant correlations with a higher percentage of inorganic arsenic and a lower percentage of DMA in urinary arsenic metabolites in Bangladesh, but not in Argentina. This suggests that the effects of *As3MT* polymorphisms on the metabolic pattern of arsenic in individuals vary among the areas or ethnic groups. They also found strong associations between two intronic polymorphisms (rs3740400 and rs1046778) and *As3MT* gene expression,⁴⁷⁾ suggesting that *As3MT* polymorphisms may also be involved in altered levels of gene expression.

Besides the polymorphisms in the *As3MT* gene, the polymorphisms in *GST* and *PNP*, which catalyze the reduction of As(V), and in *MTHFR* (methylene tetrahydrofolate reductase) and *MTR* (5-methyltetrahydrofolate-homocysteine methyltransferase), which are involved in SAM metabolism, have been reported to show significant associations with arsenic metabolism.^{38,40,48)} In addition, the polymorphism in the *CYP17A1* gene, which is located upstream from the *As3MT* gene on

chromosome 10, was reported to have a significant association with arsenic metabolism.^{49,50)} Because *CYP17A1* catalyzes sex-steroid biosynthesis, this association might be implicated in the gender difference in arsenic metabolism.^{27,40,51)}

4. ALTERNATIVE SPLICING OF *AS3MT* (FIG. 2)

We recently found two splicing variants of the human *As3MT* gene: exon-3 skipping form ($\Delta 3$) and exons-4 and -5 skipping forms ($\Delta 4,5$).⁵²⁾ A premature stop codon at exon-4 appeared in the $\Delta 3$ form of *As3MT*, whereas the $\Delta 4,5$ form of *As3MT* yielded a 31.1 kDa *As3MT* protein. When we compared the methyltransferase activity of wild-type and $\Delta 4,5$ *As3MT* recombinant proteins, a clear peak of MMA(V) was detected after the incubation of As(III) with the wild-type *As3MT* recombinant protein, but not with the $\Delta 4,5$ *As3MT* protein. In addition, COS-7 cells transfected with $\Delta 4,5$ *As3MT* cDNA did not convert As(III) to MMA(V) or DMA(V). To examine the frequency of *As3MT* splicing forms in cultured human cells, we analyzed mRNA expression of *As3MT* in HepG2, A549, HL60, K562, and HEK293 cells. The results indicated that there is a large variation in the expression of the wild-type and $\Delta 4,5$ forms of *As3MT* mRNA among cell lines. We observed clear expression of the $\Delta 4,5$ forms of *As3MT* mRNA in HL60 and K562 cells. These data suggest that the expression pattern of splicing variants of the *As3MT* gene may affect the capacity of arsenic methylation in cells. Further studies are required to test whether the splicing variants of *As3MT* exist in individuals chronically exposed to arsenic.

5. FUTURE INVESTIGATIONS

In this review, we summarized the polymorphisms of the *As3MT* gene reported to affect *As3MT* gene expression and the metabolic pattern of arsenic as well as alternative splicing of the *As3MT* gene found to affect *As3MT* activity. We consider that the following outstanding questions remain to be answered.

Does *As3MT* Catalyze Second Methylation Step *in Vivo*?

The mutagenesis from Met to Thr at 287 in human *As3MT* protein increased methyltransferase activity *in vitro* studies.

Because As3MT catalyzes the sequential methylation of arsenic to yield DMA, it was suspected that the populations have polymorphism of the *As3MT* gene, rs11191439 (Met287Thr) show significant association with a higher percentage of DMA. However, significant association was found in a higher percentage of MMA, but not of DMA. This makes us question whether As3MT really catalyzes second methylation step *in vivo*.

Is Alternative Splicing of As3MT Associated with Adverse Health Effects by Chronic Exposure to Arsenicals?

We found a lack of methylation activity of As(III) in the spliced form of the As3MT protein. We consider that the frequency of alternative splicing in the *As3MT* gene in individuals at the area of chronic arsenic exposure might be associated with the metabolic pattern of arsenic and with the adverse health effects by exposure to arsenicals.

Is It Possible to Measure the Enzymatic Activity of As3MT in Human Tissues? The arsenic metabolic pattern in urine has been utilized as a surrogate indicator of an individual's ability to methylate arsenic. The relationships among *As3MT* gene polymorphism, urinary metabolic patterns of arsenic, and As3MT activity will be elucidated in greater detail if As3MT activity can be measured in human blood samples using a highly sensitive apparatus for arsenic speciation.

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