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Effect of dietary treatment with dimethylarsinous acid (DMA^{III}) on the urinary bladder epithelium of arsenic (+3 oxidation state) methyltransferase (As3mt) knockout and C57BL/6 wild type female mice

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ABSTRACT

Chronic exposure to inorganic arsenic (iAs) is carcinogenic to the human urinary bladder. It produces urothelial cytotoxicity and proliferation in rats and mice. DMA^{V}, a major methylated urinary metabolite of iAs, is a rat bladder carcinogen, but without effects on the mouse urothelium. DMA^{III} was shown to be the likely urinary metabolite of DMA^{V} inducing urothelial changes and is also postulated to be one of the active metabolites of iAs. To evaluate potential DMA^{III}-induced urothelial effects, it was administered to As3mt knockout mice which cannot methylate arsenicals. Female C57BL/6 wild type and As3mt knockout mice (10/group) were administered DMA^{III}, 77.3 ppm in water for four weeks. Urothelial effects were evaluated by light and scanning electron microscopy (EM) and immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation. EM findings were rated 1–5, with higher rating indicating greater extent of cytotoxicity visualized. DMA^{III} significantly increased the BrdU labeling index, a ratio of BrdU labeled cells to non-labeled cells, in the treated knockout group compared to control and wild type treated groups. DMA^{III} induced simple hyperplasia in more knockout mice (4/10) compared to wild type mice (2/10). All treated knockout mice had more and larger intracytoplasmic granules compared to the treated wild type mice. Changes in EM classification were not significant. In conclusion, DMA^{III} induces urothelial toxicity and regenerative hyperplasia in mice and most likely plays a role in inorganic arsenic-induced urothelial changes. However, DMA^{V} does not induce hyperplasia in mice, suggesting that urinary concentrations of DMA^{III} do not reach cytotoxic levels in DMA^{V}-treated mice.

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1. Introduction

Inorganic arsenic (iAs) is a known human carcinogen producing tumors in urinary bladder, liver, lung, and skin (National Research Council, 1999, 2001). This metalloid occurs mainly in the +5 (arsenate) or +3 (arsenite) oxidation states, and is the 20th most abundant chemical in the earth's crust (IARC, 2004). Because it is present in soils, it can be leached into ground water. As-contaminated ground water is used as drinking water by populations worldwide and constitutes an important source of exposure to this toxicant that is associated with increased incidence of both cancer and non-cancer health effects (Chiou et al., 1995). Although dose–response relations for these adverse health effects are not fully understood (Lamm et al., 2006), it is clear that arsenic exposure contributes to tumor risk (National Research Council, 1999, 2001).

Elucidating the processes underlying the adverse health effects of chronic arsenic exposure is complicated by the extensive metabolism that follows ingestion or inhalation of iAs. Mono-, di-, and trimethylated metabolites are found in tissues and excreta of animals exposed to iAs. The formation of methylated arsenicals is catalyzed in many mammalian species by arsenic (3+ oxidation state) methyltransferase (As3mt) (Thomas et al., 2007). In the Challenger scheme, a series of reactions starting with arsenite produces methylarsonic acid (MMA^{V}) which is reduced to methylarsonous acid (MMA^{III}) that is converted to dimethylarsinic acid (DMA^{V}) and reduced to form dimethylarsinous acid (DMA^{III}). In some species, particularly rats and to lesser extent other rodents, DMA^{III} is further methylated to form trimethylarsine oxide ($TMA^{V}O$) (Adair et al., 2007). $TMA^{V}O$ is only formed in humans when there is very high exposure to inorganic arsenic (Aposhian, 1997; Cohen et al., 2006; Thomas, 2007). These methylated metabolites of iAs exert specific

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toxicities in animal models. For example, DMA^V produces urinary bladder tumors in rats but is not carcinogenic in mice (Arnold et al., 2006; Wei et al., 1999, 2002). DMA^{III} is thought to be the reactive metabolite that produces changes in the urothelium associated with exposure to DMA^V. Development of urinary bladder tumors in DMA^V-exposed rats is possibly related to regenerative proliferation caused by the cytotoxic effect of the reactive metabolite DMA^{III} (Cohen et al., 2006, 2007; Waalkes et al., 2006). In contrast, MMA^V is negative as a carcinogen in rat and mouse bioassays (Arnold et al., 2006) but is a transplacental carcinogen in the mouse (Tokar et al., 2012). Thus, because the metabolism of iAs forms methylated metabolites that are more reactive and toxic than the parent compound (Cohen et al., 2002; Petrick et al., 2000; Styblo et al., 2000), it is likely that the methylated arsenicals contribute to the spectrum of adverse health effects associated with chronic exposure to iAs (Cohen et al., 2006).

Given the central role of arsenic methylation in the formation of metabolites that mediate the toxic and carcinogenic effects associated with chronic exposure to iAs, manipulation of the capacity to methylate arsenic is one approach to understanding underlying molecular processes. Studies in As3mt knockout mice have shown that this enzyme plays a central role in the control of the disposition and retention of arsenic in arsenate-treated mice (Drobna et al., 2009; Hughes et al., 2010). Studies of the effects of arsenite exposure in wild-type and As3mt knockout mice have shown that changes in the urothelium are qualitatively similar but more pronounced in knockout mice than in wild-type mice (Chen et al., 2011; Yokohira et al., 2010, 2011). The genotypic differences in the effects of arsenite exposure on the urothelium can largely be explained by the high retention of iAs in the urinary bladders of As3mt knockout mice.

The present study examines effects on urothelial structure after repeated exposure of wild-type and As3mt knockout mice to DMA^{III}, the putative active metabolite of DMA^V. Here, the As3mt genotype affected morphological changes in the urothelium with a greater response in As3mt knockout mice than in wild-type mice. Evidence of regenerative proliferation in mice of either As3mt genotype suggests that cytotoxicity and cell regeneration could plausibly underlie urinary bladder tumorigenesis in the mouse.

2. Materials and methods

2.1. Chemicals

Dimethylarsinous iodide (DMA^{III}) was obtained from Eurolabs Ltd. (Cheshire, UK) and stored desiccated at 2–8 °C. The purity of the chemical was greater than 95%. Bromodeoxyuridine (BrdU) was purchased from (Sigma–Aldrich, St. Louis, MO). Nembutal (Lundbeck, Deerfield, IL) was obtained from the Nebraska Medical Center pharmacy.

2.2. Animals

2.2.1. As3mt knockout mice

Four female and two male mice homozygous for the disrupted As3mt gene (Drobna et al., 2009) were obtained from Dr. David Thomas (U.S. Environmental Protection Agency, Research Triangle Park, NC). Exons 3 through 5 were deleted by homologous recombination to generate the As3mt KO homozygous mice. The altered gene was introduced and maintained in strain 129S6 mice before being introduced into strain C57BL/6 mice by marker-assisted accelerated backcrossing performed by Charles River Laboratories (Wilmington, MA) to produce homozygous $As3mt^{-/-}$ mice. The mice were fertile, so brother/sister matings were used to maintain the homozygous As3mt KO genotype. Twenty As3mt KO mice from the F6 generation, approximately 11 weeks old, were transferred from the breeding colony at the University of Nebraska Medical Center, Omaha, NE.

2.2.2. Wild type mice

Twenty female C57BL/6 mice (Charles River Breeding Laboratories, Kingston, NY) approximately 11 weeks old at the time of arrival were used in the study.

2.3. Animal experiment

All animals were placed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility and quarantined for 7 days prior to treatment. The level of care provided met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The animals were housed in polycarbonate cages (5 per cage) with micro-isolator tops and dry corncob bedding. Nestlets (Ancare, Bellmore, NY) were placed inside the cages for environmental enrichment. Animals were maintained at approximately 22 °C and 50% relative humidity with a 12-h light/dark schedule, and were provided with pelleted Purina 5002 diet (Dyets Inc., Bethlehem, PA) and water (hyperchlorinated, RO) ad libitum throughout the study.

Animals were randomized into two groups per genotype. One group in each genotype served as the control and the other group was administered 77.3 ppm dimethylarsinous iodide (equivalent to 25 ppm elemental arsenic that previously produced urothelial hyperplastic changes in these mice after 4 weeks of treatment (Yokohira et al., 2010, 2011)) in drinking water by injecting into water bags (Hydropac® bags, Lab Products, Seaford, DE). Freshly prepared DMA^{III} was injected into bags just before replacement. The water bags in both the control and DMA^{III}-treated groups were replaced once every two days.

The animals were treated for four weeks. One hour before sacrifice the mice were injected with BrdU, 100 mg/kg intraperitonially. At sacrifice the urinary bladders were inflated in situ with Bouin's fixative, removed and placed in Bouin's fixative along with a small section of intestine. After fixation, bladders were weighed, observed macroscopically and divided in half longitudinally. One half of the bladder was cut into longitudinal strips, embedded into paraffin with a slice of intestine, sectioned and stained with hematoxylin and eosin for histopathological examination. A diagnosis of mild hyperplasia was made when there were four to five cell layers in the bladder epithelium and moderate hyperplasia when six to eight layers were present. Histopathological diagnosis is made without knowledge of treatment group. The other half of each bladder was processed for examination by scanning electron microscopy (EM) and classified in one of five categories as previously described (Cohen et al., 1990). The categories have the following characteristics: class 1 bladders contain polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis, especially in the dome of the bladder; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal animals show class 1 or 2, or occasionally class 3. Kidneys were removed, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin and examined histopathologically.

Unstained bladder and intestinal tissue slides were processed for immunohistochemical detection of BrdU (Cohen et al., 2007). Intestinal tissue served as a positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:200. The BrdU-labeled cells in at least 3000 urothelial cells were counted to determine a labeling index. Microscopic examination and counting of BrdU-labeled cells was done on blinded slides.

2.4. Statistics

Comparison of all data collected on body weights, food and water consumption, bladder weights, and the labeling index was performed by the SAS general linear models procedure and Duncan's multiple range test. Selected intra-genotype and inter-genotype comparisons for control and treated groups were performed. All means are accompanied by calculation of standard errors. Histology results were analyzed using Fisher's Exact test (2-tail).

3. Results

3.1. General findings

All animals including treated KO mice showed no adverse signs, and none died during the experimental period. There was no significant difference in body weight or food and water intake between respective groups. There was a statistically significant but small increase of the relative kidney weight in treated compared to the control knockout mice.

3.2. Histopathology

Simple hyperplasia was observed in 4 of the 10 DMA^{III}-treated knockout mice and only 2 of the 10 treated wild-type mice, with none in the control knockout or wild-type mice (Fig. 1C, F and Table 1). The superficial layer of the urothelium contained eosinophilic intracytoplasmic granules (Suzuki et al., 2008)

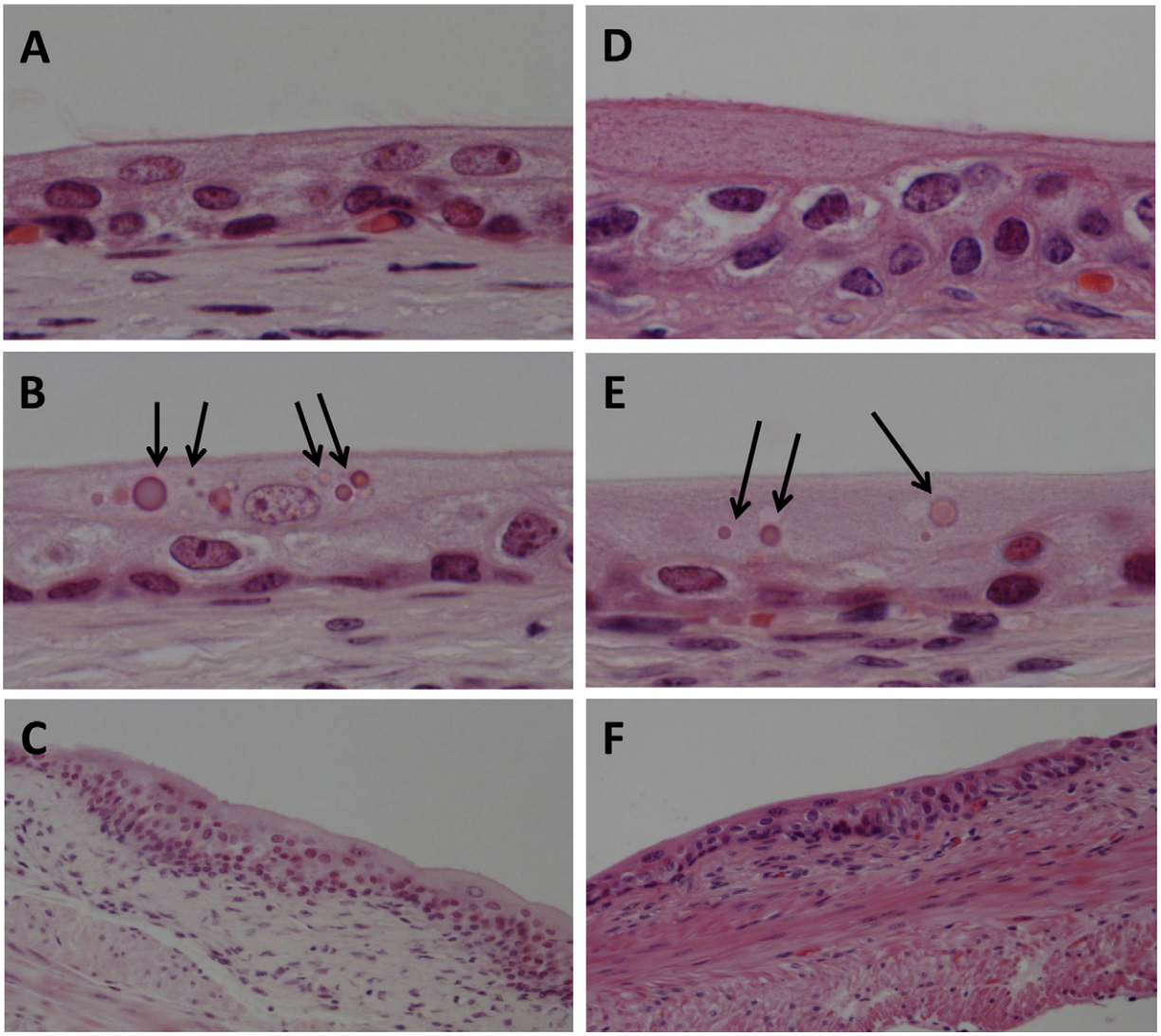

Figure 1. DMA^{III}-induced histopathological changes in the urothelium. Histopathological examination of the urothelium by hematoxylin and eosin staining showed normal urothelium in knockout control mice (A) and the presence of eosinophilic intracytoplasmic granules in the superficial layer of the urothelium (B) and simple hyperplasia (C) in DMA^{III}-treated knockout mice. Similarly, wild-type control mice showed normal urothelium (D) and DMA^{III}-treated wild-type mice showed the presence of eosinophilic intracytoplasmic granules (E) and simple hyperplasia (F). The granules in both knockout and wild-type mice appeared to be of various sizes and numbers per cell (B, E, arrows). Magnification 1000× (A, B, D and E) and 200× (C and F).

in all DMA^{III}-treated mice (Fig. 1). Granules were round and of varying sizes, with multiple granules occurring frequently in individual cells (Fig. 1B, E). The granules were slightly smaller and fewer in treated wild-type mice compared to treated knockout mice. None of the control mice had granules (Fig. 1A, D). The granules were present in the DMA^{III}-treated bladders whether they had simple hyperplasia or were normal. The kidneys showed no remarkable changes by histopathological examination, except one mouse in the knockout-treated group showed focal, chronic interstitial inflammation.

Table 1
Effects of DMA^{III} treatment on the urinary bladder epithelium in mice. Mice were administered 77.3 ppm (equivalent of 25 ppm arsenic) through drinking water for 4 weeks. The urothelium was investigated for hyperplasia by hematoxylin and eosin staining, BrdU labeling index by immunohistochemistry, and visualization by scanning electron microscopy (EM).

Group	Treatment	Strain	Histopathology		BrdU labeling index (%) Mean ± S.E. (*n*)	SEM classification			
			Normal	Simple hyperplasia		1	2	3	4
1	Control	KO	10	0	0.11 ± 0.06 (9)	3	5	1	1
2	77.3 ppm DMA^{III}	KO	6	4	0.75 ± 0.27 (9)[a,b]	2	4	4	0
3	Control	WT	10	0	0.20 ± 0.14 (9)	3	5	2	0
4	77.3 ppm DMA^{III}	WT	8	2	0.08 ± 0.03 (10)	5	4	1	0

[a] Significantly different compared to KO control group, $p < 0.05$.
[b] Significantly different compared to the WT DMA^{III} group, $p < 0.05$.

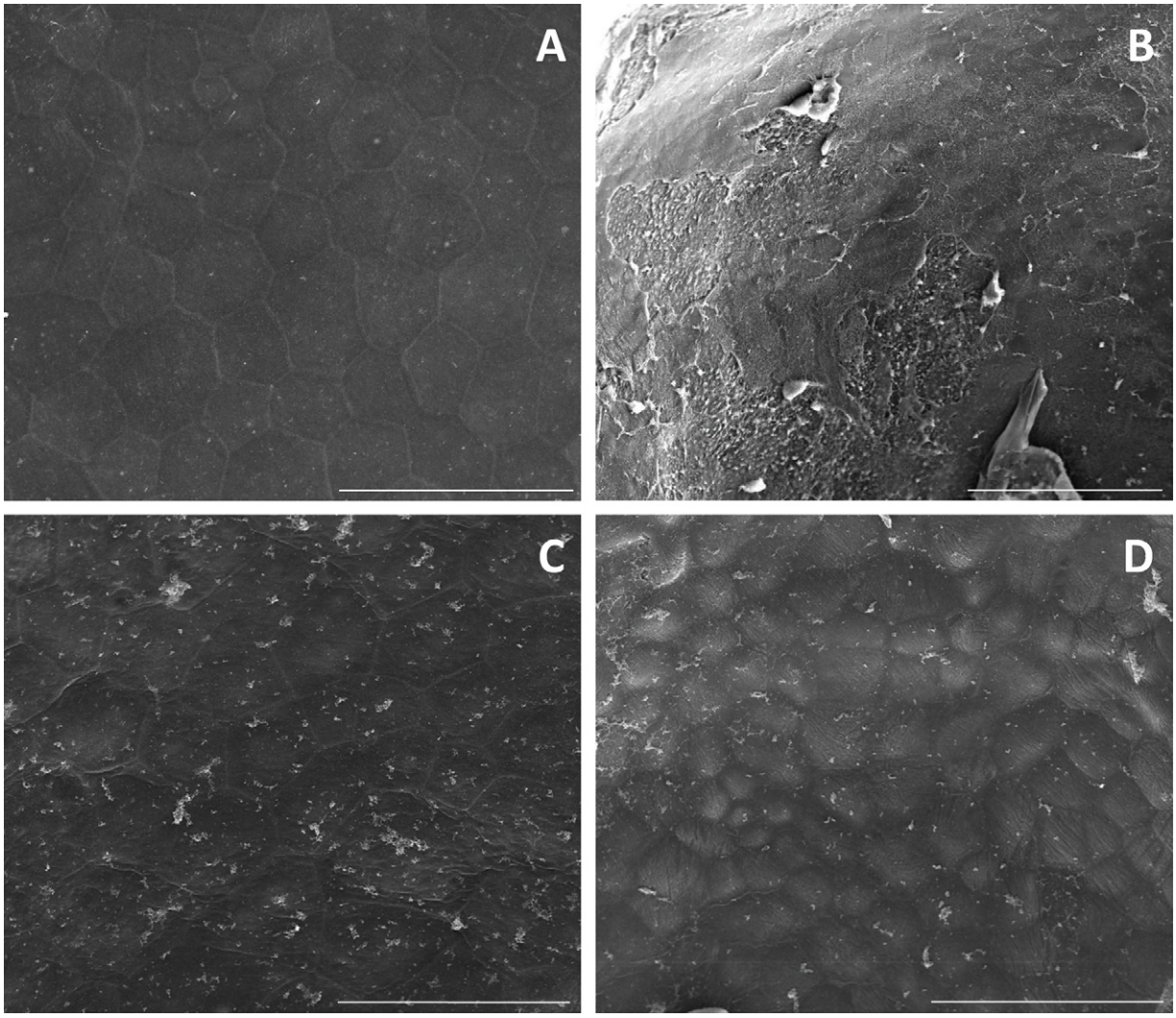

Figure 2. Scanning electron microscopy (EM) examination of the bladder urothelium. Urothelium of control knockout mice was normal in appearance (A, bar = 300 μm) whereas necrosis and exfoliation of the superficial cells was present in the bladder urothelium from DMA^{III}-treated As3mt knockout mice (B, bar = 500 μm). In the wild-type mice, the urothelium was normal in both control (C, bar = 200 μm) and DMA^{III}-treated (D, bar = 300 μm) groups.

3.3. Scanning electron microscopy

Although the changes visualized by EM were not statistically significant, there was a clear trend for DMA^{III}-induced cytotoxicity in the treated knockout mice compared to other groups (Table 1 and Fig. 2). The DMA^{III}-treated knockout mice had only 2 bladders with class 1 changes and 4 bladders with class 2 changes, which are considered normal for rodents (Cohen et al., 1990). However, four bladders in the same group showed class 3 changes, which contain numerous small foci of superficial urothelial necrosis (Fig. 2B), whereas the knockout control group had 3 and 5 bladders with class 1 and class 2 changes (Fig. 2A), respectively, and only one each with class 3 and class 4 changes. Among the wild type, DMA^{III}-treated mice there was no significant cytotoxicity in the bladder by EM compared to the control group (Fig. 2C, D).

3.4. BrdU labeling index

The DMA^{III}-treated knockout mice had a significantly increased BrdU labeling index compared to the knockout control group and also compared to the DMA^{III}-treated wild-type group of mice.

4. Discussion

Results of the present study showed that DMA^{III} administration in drinking water for four weeks induced urothelial cytotoxicity and regenerative urothelial cell proliferation in As3mt knockout mice which was somewhat greater compared to the treated wild-type mice. DMA^{III} induced formation of intracytoplasmic granules in the superficial urothelial cell layer in both wild-type and knockout mice. There were no instances of systemic toxicity in wild-type or knockout mice.

DMA^{III} induced simple hyperplasia in more knockout mice compared to wild-type mice, however the increase was not statistically significant. This was further demonstrated by a significant increase in BrdU labeling index in treated knockout mice, indicating increased cell proliferation leading to simple hyperplasia; the labeling index was not increased in wild type mice. These results are similar to what we have observed for arsenite-administered As3mt knockout compared to wild-type mice (Yokohira et al., 2010, 2011). However, in the As3mt knockout mice, arsenite induced more pronounced systemic toxicity at comparable doses than in wild-type mice (Yokohira et al., 2010, 2011). This was attributed to decreased clearance of arsenite in the As3mt knockout

mice (Drobna et al., 2009; Yokohira et al., 2010, 2011). Methylation enhances the clearance of arsenic through urine (Cohen et al., 2006; Kenyon et al., 2008), but in As3mt knockout mice, arsenic is cleared slowly and accumulates in the tissues (Chen et al., 2011). In the current study, there was a significant difference between the DMA^{III}-induced effects in As3mt knockout mice compared to wild-type mice, suggesting either that DMA^{III} is not cleared as efficiently as in the wild-type mice, or loss of As3mt function makes the urothelium more susceptible to DMA^{III}. The first explanation is more likely. Mice are known to methylate DMA to $TMA^{V}O$ but only to a limited extent (Cohen et al., 2006). DMA ($DMA^{III} + DMA^{V}$) remains the major metabolite excreted in urine (Cohen et al., 2006). This suggests that wild-type mice may further methylate a significant quantity of DMA^{III} to $TMA^{V}O$, which is less reactive and less cytotoxic and will reduce the net DMA^{III} excreted in the urine. Metabolic and kinetic analyses are required to distinguish these possibilities.

DMA ($DMA^{III} + DMA^{V}$) is the major metabolite in people exposed to inorganic arsenic and also in rats and mice exposed to arsenate, arsenite, MMA^{V} and DMA^{V} (Cohen et al., 2006). Among the arsenicals studied in 2-year bioassays DMA^{V} was carcinogenic to the bladder in rats but not mice (Arnold et al., 2006). Arsenate and arsenite are carcinogenic to mice, although the carcinogenic effect on the urothelium is limited (Cohen et al., 2007; Tokar et al., 2010; Waalkes et al., 2006). Interestingly, MMA^{V} was not carcinogenic to either mice or rats (Arnold et al., 2003). In short term studies in rats, orally administered arsenate, arsenite and DMA^{V} induced urothelial hyperplasia, the preneoplastic proliferative lesion of the urothelium (Cohen et al., 2007). Arsenate and arsenite also induced urothelial hyperplasia in mice, but DMA^{V} did not (Arnold et al., 2006; Cohen et al., 2006). The data in the current study show that DMA^{III} also induces simple hyperplasia of the urothelium in mice, suggesting a role for DMA^{III} in the inorganic arsenic-induced pre-neoplastic changes and carcinogenic effects.

We have previously reported the detection of numerous intracytoplasmic and intranuclear granules in As^{III}-treated mice (Suzuki et al., 2008; Yokohira et al., 2010, 2011). In this study, DMA^{III} also induced intracytoplasmic granules in both As3mt knockout and wild-type mice. These granules were confined to the superficial layer and were seen in bladders with or without simple hyperplasia. Our earlier study showed the presence of granules in all layers of the urothelium in inorganic arsenic-administered to As3mt knockout mice, and the severity of granule formation was dose-dependent (Yokohira et al., 2010, 2011). Compared to the arsenic-equivalent concentration of inorganic arsenic used in the previous studies, the severity of granule formation by DMA^{III} was less in terms of both number of granules and the layers of urothelium affected. This may be due to faster clearance of pre-methylated DMA^{III} compared to non-methylated inorganic arsenic. The significance or the role of these granules in arsenic-induced toxicity is still unclear.

The current study demonstrated that DMA^{III} induces urothelial cytotoxicity and regenerative proliferation in As3mt knockout mice and to a lesser extent in the wild-type mice, unlike DMA^{V} that did not induce any urothelial changes in wild type mice (Arnold et al., 2006; Cohen et al., 2006). Similar to inorganic arsenic, DMA^{III} induced formation of intracellular granules in the urothelium of both As3mt knockout and wild-type mice, though to a lesser extent than inorganic arsenic. These results support our hypothesis that urothelial toxicity is induced by reactive arsenicals, including DMA^{III}, which is a highly reactive, trivalent methylated arsenical. Cytotoxicity is followed by regenerative proliferation resulting in hyperplasia and ultimately possibly tumors.

Conflict of interest statement

None declared.

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