Characterization of Intracellular Inclusions in the Urothelium of Mice Exposed to Inorganic Arsenic

Puttappa R. Dodmane,* Lora L. Arnold,* David E. Muirhead,* Shugo Suzuki,† Masanao Yokohira,‡ Karen L. Pennington,* Bhavana J. Dave,§ Xiufen Lu,¶ X. Chris Le,∥ and Samuel M. Cohen*¶†‡

*Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-3135; †Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi 467–8601, Japan; ‡Department of Pathology and Host-defense, Kagawa University Faculty of Medicine, Miki-cho, Kita-gun, Kagawa 761–0795, Japan; ¶Human Genetics Laboratories, Munroe Meyer Institute for Genetics and Rehabilitation (MMI), University of Nebraska Medical Center, Omaha, Nebraska 68198-5440; ∥Analytical and Environmental Toxicology Division, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta T6G 2G3, Canada; and ¶Havlík-Wall Professor of Oncology

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Inorganic arsenic (iAs) is a known human carcinogen at high exposures, increasing the incidences of urinary bladder, skin, and lung cancers. In most mammalian species, ingested iAs is excreted mainly through urine primarily as dimethylarsinic acid (DMA\(^3\)). In wild-type (WT) mice, iAs, DMA\(^3\), and dimethylarsinous acid (DMA\(^\text{III}\)) exposures induce formation of intramitochondrial urothelial inclusions. Arsenite (iAs\(^\text{III}\)) also induced intranuclear inclusions in arsenic (+3 oxidation state) methyltransferase knockout (As3mt KO) mice. The arsenic-induced formation of inclusions in the mouse urothelium was dose and time dependent. The inclusions do not occur in iAs-treated rats and do not appear to be related to arsenic-induced urothelial cytotoxicity. Similar inclusions in exfoliated urothelial cells from humans exposed to iAs have been incorrectly identified as micronuclei. We have characterized the urothelial inclusions using transmission electron microscopy (TEM), DNA-specific 4′,6-diamidino-2-phenylindole (DAPI), and non-DNA-specific Giemsa staining and determined the arsenical content. The mouse inclusions stained with Giemsa but not with the DAPI stain. Analysis of urothelial mitochondrial- and nuclear-enriched fractions isolated from WT (C57BL/6) and As3mt KO mice exposed to arsenate (iAs\(^\text{V}\)) for 4 weeks showed higher levels of iAs\(^\text{V}\) in the treated groups. iAs\(^\text{III}\) was the major arsenical present in the enriched nuclear fraction from iAs\(^\text{V}\)-treated As3mt KO mice. In conclusion, the urothelial cell inclusions induced by arsenicals appear to serve as a detoxifying sequestration mechanism similar to other metals, and they do not represent micronuclei.

Key Words: micronuclei; granules; carcinogenesis; genotoxicity; intramitochondrial; intranuclear.

Arsenic is a metalloid element with atomic number 33. Chronic exposure to high doses of inorganic arsenic (iAs) (arsenate [iAs\(^\text{V}\)] and arsenite [iAs\(^\text{III}\)]) causes cancer and noncancerous effects in humans (IARC, 2004; National Research Council, 2001). iAs has been classified as a group I carcinogen since 1989 (IARC, 2004). Chronic ingestion occurs mainly through drinking water contaminated with iAs either from geologic formations or from human activity, but also occurs from food and by inhalation. Chronic high exposures to iAs ingestion leading to increased rates of urinary bladder, skin, and lung cancer are a significant public health problem in several countries including Taiwan, Mongolia, Bangladesh, India, China, Chile, Argentina, and Mexico. Certain parts of the United States have relatively high levels of arsenic in drinking water, but not as high as in those other countries (Gronberg, 2011).

In most organisms, iAs is metabolized by an alternating series of reductions of pentavalent forms to trivalent forms followed by sequential oxidative methylation. This metabolism yields iAs\(^\text{III}\), monomethylarsonic acid (MMA\(^\text{V}\)), monomethylarsonous acid (MMA\(^\text{III}\)), dimethylarsinic acid (DMA\(^\text{V}\)), dimethylarsinous acid (DMA\(^\text{III}\)), and trimethylarsine oxide (TMA\(^\text{O}\)) (Le et al., 2000; Vahter, 2002). DMA\(^\text{V}\) is the most abundant metabolite excreted in urine of humans and rodents. Among these arsenicals, trivalent arsenicals have been shown in vitro to be highly reactive and considerably more cytotoxic compared with pentavalent forms, especially for the methylated arsenicals (Cohen et al., 2006). These trivalent forms are believed to play a critical role in iAs-induced toxicity and carcinogenicity (Cohen et al., 2007; Dodmane et al., 2013; Hughes et al., 2011).

As previously reported (Arnold et al., 2006; Dodmane et al., 2013; Suzuki et al., 2008b; Yokohira et al., 2010, 2011), intracellular eosinophilic inclusions are observed in the superficial layer of the urothelium of mice exposed to iAs\(^\text{III}\), iAs\(^\text{V}\), DMA\(^\text{III}\), or DMA\(^\text{V}\). These inclusions were present in all layers of the urothelium in iAs\(^\text{III}\)-treated arsenic (+3 oxidation state) methyltransferase knockout (As3mt KO) mice, which are unable to
methylate iAs. The inclusions were located in mitochondria and nuclei in As3mt KO mice and in mitochondria in wild-type (WT) mice. These inclusions do not appear to play a role in the urothelial toxicity of arsenicals because they do not occur in rats treated with arsenicals even though urothelial toxicity occurs in both rats and mice. It is possible that they act as a protective sequestering mechanism as seen with other metals (Brown et al., 1985; Masci & Bongarzone, 1995; Gonick, 2011).

In humans, the presence of cytoplasmic inclusions in exfoliated urothelial cells in urine was reported in epidemiological studies in populations exposed to high levels of arsenic in drinking water (Basu et al., 2002; Ghosh et al., 2008). These inclusions have been labeled as micronuclei, often based on staining with the nonspecific Giemsa stain (Basu et al., 2002) or staining with centromere-specific stains in which the majority of the inclusions were centromere negative (Ghosh et al., 2008; Marchiset-Ferlay et al., 2012; Moore et al., 1997). We have recently reported the presence of intracytoplasmic eosinophilic inclusions, similar to those in mice, in exfoliated cells in urine from promyelocytic leukemia (PML) patients treated with arsenic trioxide (ATO) (Wedel et al., 2013). These inclusions in the urothelial cells from ATO-treated patients do not stain with the DNA-specific stain 4′,6-diamidino-2-phenylindole (DAPI), indicating that the inclusions are not micronuclei, despite their similar morphology to the inclusions claimed to be micronuclei by other researchers. Furthermore, observations by transmission electron microscopy (TEM) showed that the inclusions in mice or humans were not morphologically related to micronuclei (Suzuki et al., 2008a; Wedel et al., 2013).

The composition of the inclusions in the mouse urothelium is not known. In our previous study (Suzuki et al., 2008a), iAsIII was the major species identified in the mitochondrial fraction isolated from urothelial cells that were collected from the iAsIII-treated mice, suggesting that the inclusions were a storage depot for arsenic.

The urothelial inclusions found in arsenical-treated mice were characterized using TEM, DNA-specific (DAPI) and non-DNA-specific (Giemsa) stains. Speciation and quantitation of the arsenical content of enriched urothelial mitochondrial and nuclear fractions isolated from iAsIII-treated WT and As3mt KO mice were determined.

MATERIALS AND METHODS

Chemicals

Sodium arsenite (NaAsO₂), ≥ 99% pure, and sodium arsenate (Na₃H₂AsO₄·7H₂O), 99.7% pure, were purchased from Sigma-Aldrich (St Louis, Missouri). Focus SubCell (G-Biosciences, St Louis, Missouri) was used to isolate and enrich subcellular fractions.

Animals

WT C57BL/6 mice were purchased from Charles River Breeding Laboratories (Raleigh, North Carolina, for Experiment 1; Portage, Michigan, for Experiment 3). The As3mt KO mice were bred from 4 female and 2 male mice homozygous for the disrupted As3mt gene (Droba et al., 2009) obtained from Dr David Thomas (U.S. Environmental Protection Agency, Research Triangle Park, North Carolina). 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 bred into the C57BL/6 strain of mice by marker-assisted accelerated backcrossing performed by Charles River Laboratories (Wilmington, Massachusetts) to produce homozygous As3mt−/− mice. The mice were fertile, so brother/sister matings were used to maintain the homozygous As3mt KO genotype.

All animals were placed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and quarantined for at least 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). Experimental protocols were approved by the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC). Animal husbandry included housing in polycarbonate cages (5 per cage) with micro-isolator tops and dry corncob bedding. Nestlets (Ancare, Bellmore, New York) 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, Pennsylvania) and hyperchlorinated, reverse osmosis water or tap water ad libitum throughout the study. Total arsenic content of the drinking water was < 8 ppb according to analyses by the Omaha Metropolitan Utilities District, and the diet had < 0.2 ppm total arsenic according to analyses provided by Purina.

Experiment 1

To evaluate iAsII-induced urothelial inclusions and to determine arsenic speciation and quantification in enriched mitochondrial and nuclear fractions, 80 female WT and 80 female As3mt KO mice approximately 10–12 weeks of age were divided randomly by a weight stratification method (Martin et al., 1984) into 2 groups of 40 mice each per genotype. One group in each genotype was administered 104 ppm sodium arsenate (equivalent to 25 ppm elemental arsenic) in drinking water for 4 weeks by injecting freshly prepared sodium arsenate into water bags (Hydroup, Lab Products, Seaford, Delaware) twice per week. After 4 weeks, 30 animals from each group were sacrificed, and urothelial cells were removed mechanically by scraping and stored in PBS at −80°C. The remaining 10 animals were used to evaluate urothelial changes as reported earlier (Cohen et al., 2007).

Sacrifice and tissue processing. All animals were sacrificed by an overdose of Nembutal (150 mg/kg body weight, IP) followed by exsanguination after removal of specific tissues. Urinary bladders used for collection of urothelial cells were inflated in situ while the mice were still alive and under deep anesthesia with cold PBS. After removal, the bladders were inverted on wood applicator sticks and the epithelia scraped gently with the edge of a scalpel blade. The urothelial cells were removed by swirling the scalpel blade in microcentrifuge tubes containing PBS with protease inhibitor (Sigma) and kept at approximately −80°C until processed for purification of enriched mitochondrial and nuclear fractions. To determine efficiency of removal of the urothelium, the remaining bladder tissue was fixed in Bouin’s fixative, processed for paraffin embedding, stained with H&E, and examined by light microscopy. Urinary bladders used for paraffin embedding were inflated and fixed with Bouin’s fixative, along with a piece of small intestine. The paraffin-embedded tissue sections were stained with various stains described below.

Enrichment of mitochondria and nuclei. Mitochondrial- and nuclear-enriched fractions were isolated using a modification of the method developed by Nagata et al. (2010). Briefly, urothelial cells collected from each group in Experiment 1 were thawed and passed through a 35-µm cell strainer (BD Biosciences) to lyse the cells. The whole cell lysate was centrifuged at 3000rpm (Eppendorf 5402) for 5 min to separate nuclei from the cytoplasmic
fraction. The crude nuclear fraction was further purified using a sucrose gradient (1.12 M sucrose, 3 mM MgCl$_2$, 10 mM Tris-HCl, pH 7.4) and centrifuged for 30 min at 14000 rpm in an Eppendorf 5402 centrifuge (Eppendorf, Hauppauge, New York). The sediment was further washed in sucrose solution (250 mM sucrose, 5 mM MgCl$_2$, 10 mM Tris-HCl, pH 7.4). The cytoplasmic fraction was centrifuged at 14 000 rpm for 10 min. The sediment was retained, washed in sucrose buffer (1 M sucrose, 1 mM MgCl$_2$, 10 mM Tris-HCl, pH 7.4) and dissolved in Mitochondrial Storage Buffer (G-Biosciences). A portion of each enriched fraction was fixed in glutaraldehyde/paraformaldehyde solution and processed for TEM examination. The remaining portions of each fraction were frozen at −80°C until processing for quantitation and speciation of arsenicals.

Quantitation and speciation of arsenicals. The frozen samples of enriched mitochondria and nuclei were thawed and centrifuged at 4000 rpm for 10 min at 4°C (Micromax RF, Thermo IEC). The supernatant was filtered through a 0.45-µm nylon membrane and diluted with deionized water for analysis of small molecule arsenic species. For analysis of all arsenic species including macromolecular-bound species, the precipitate was mixed with RIPA solution (5 mM Tris; pH 7.5, 15 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate, and 0.01% SDS), ethanol, and dimethyl sulfoxide and sonicated for 30 min followed by dilution with deionized water. The solution was centrifuged for 10 min at 4000 rpm, and the supernatant was analyzed.

Quantitative determination of the small molecule arsenic species and macromolecular-bound arsenic species was conducted by HPLC separation coupled with ICPMS detection system. For separation of the small molecule arsenic species, the methods have been described previously (Le et al., 2000; Lu et al., 2003; Yuan et al., 2008). For separation of the macromolecular-bound arsenic species, a size exclusion column (Biosep-S 2000, 300 × 4.6 mm, Phenomenex, Torrence, California) was used. The mobile phase contained 35 mM NH$_4$HCO$_3$, 5% methanol, pH 8.5. The flow rate was 1.0 ml/min. The effluent from the post column was brought directly into the ICPMS (Agilent 7500 ce) for arsenic species detection. m/z 75 was monitored for arsenic species. Helium mode was used for interference elimination.

Because protein content of each enriched fraction was not known, the percent contribution of arsenic species to the total arsenic content of each enriched fraction was calculated to compare between the treated groups (Table 1).

Experiment 2

To evaluate inclusions in As3mt KO mice by TEM, 6 female and 4 male As3mt KO mice, approximately 31–32 weeks old, were transferred from the breeding colony and randomized (Martin et al., 1984) based on sex and weight into 2 groups of 3 female mice each and 2 male mice. One group for each sex was administered 43.3 ppm sodium arsenite (25 ppm elemental arsenic) in tap water for 2 weeks. Animals were sacrificed as described in Experiment 1 except that a glutaraldehyde/paraformaldehyde fixative was used for in situ inflation of the urinary bladder while mice were still alive and under deep anesthesia. Bladders were removed and placed in the same fixative. The bladders were processed for examination by TEM as described previously (Suzuki et al., 2008a).

Experiment 3

To evaluate urothelial recovery from inclusions after treatment and to prepare cytospin smears from urothelial scrapings, 20 female WT mice, approximately 7 weeks of age, were randomized (Martin et al., 1984) by weight into 2 groups of 10 animals each and treated with 0 ppm (control) or 173.2 ppm of sodium arsenite (100 ppm elemental arsenic) for 4 weeks. After 4 weeks, 5 mice in each group were sacrificed to assess urothelial changes by histopathology (Cohen et al., 2007) as described in Experiment 1. Except in 1 animal from the treated group, the urothelium was scraped as described in Experiment 1, and a single cell solution was prepared by passing the scraped material through a 35-µm cell strainer (BD Biosciences, Durham, North Carolina). A cytospin smear was prepared from this single cell solution, stained with Wright-Giemsa stain (referred to as Giemsa stain), and observed by light microscopy. Remaining mice were allowed to recover by administering regular water for an additional 90 days before they were sacrificed to assess the urothelial changes by histopathology as described in Experiment 1.

Staining Procedures

Paraffin-embedded bladder tissue was stained with H&E, Modified Wright-Giemsa Stain (Sigma-Aldrich), and DAPI as per the standard operating procedure in the Tissue Sciences Facility, UNMC. The H&E staining was carried out according to standard operating procedure developed for the autostainer using Hematoxylin 560MX and Eosin Y515 from Leica (Richmand, Illinois). Briefly, for Giemsa staining, the tissue sections were dehydrated in alcohol, fixed in xylene, washed in ethanol, stained with Modified Wright-Giemsa solution for 5 min, and washed. For DAPI staining, dewaxed tissue sections were dipped in DAPI solution (Sigma-Aldrich) for 5 min, washed in water, and air dried. The cytospin slide prepared from mouse urothelial scrapings using a Shandon Cytospin 3 centrifuge (Thermo Scientific, Kalamazoo, Michigan) was stained with Wright-Giemsa stain (Sigma-Aldrich) according to the standard operating procedure followed in the Department of Human Genetics Laboratory, UNMC.

### Table 1

<table>
<thead>
<tr>
<th>Enriched Fraction</th>
<th>Treatment</th>
<th>Strain</th>
<th>As$^V$</th>
<th>As$^{III}$</th>
<th>MMA</th>
<th>DMA</th>
<th>Total Arsenic (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitochondria</strong></td>
<td>Control</td>
<td>WT</td>
<td>0.08 (100)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>25 ppm As$^V$</td>
<td>WT</td>
<td>0.16 (92)</td>
<td>nd</td>
<td>nd</td>
<td>0.01 (8)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>KO</td>
<td>0.08 (100)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>25 ppm As$^V$</td>
<td>KO</td>
<td>0.15 (76)</td>
<td>0.04 (18)</td>
<td>nd</td>
<td>0.01 (6)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Nuclei</strong></td>
<td>Control</td>
<td>WT</td>
<td>0.06 (80)</td>
<td>nd</td>
<td>0.001 (1.6)</td>
<td>0.02 (19)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>25 ppm As$^V$</td>
<td>WT</td>
<td>0.12 (54)</td>
<td>0.014 (6)</td>
<td>0.003 (1.5)</td>
<td>0.09 (38)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>KO</td>
<td>0.07 (65)</td>
<td>0.004 (4)</td>
<td>0.002 (1.9)</td>
<td>0.03 (29)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>25 ppm As$^V$</td>
<td>KO</td>
<td>0.16 (28)</td>
<td>0.349 (59)</td>
<td>0.003 (0.5)</td>
<td>0.07 (12)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*Note.* Detection limit: 0.1 µg/l for all species. Abbreviation: nd, not detectable.
RESULTS

Characteristics of Inclusions in Mouse Urothelium

Repeated oral exposure of WT female mice to iAs\(^{\text{V}}\) resulted in formation of intracytoplasmic inclusions in the superficial layer of the urothelium. By H&E staining, the intracytoplasmic inclusions were eosinophilic and round, of varying sizes, with multiple inclusions frequently present per cell (Fig. 1). In contrast to the WT mice (Fig. 1B), intracytoplasmic inclusions were present in all layers of the urothelium (Figs. 1C and 2B) and in nuclei (Figs. 1C and 2D) of As3mt KO mice treated with iAs\(^{\text{V}}\) or iAs\(^{\text{III}}\). When WT and As3mt KO mice were treated with equivalent doses of iAs\(^{\text{V}}\) (Figs. 1B and C), the number of inclusions was higher in the As3mt KO mice compared with WT mice.

Examination of Mouse Urothelial Inclusions by TEM

TEM examination of intact urothelial sections of iAs\(^{\text{III}}\)-treated mice clearly showed the presence of homogenous, round inclusions in the mitochondria (Figs. 2B and C) and irregular shaped but homogenous intranuclear inclusions in the female As3mt KO mice (Figs. 2B and D). Male As3mt KO mice did not show intranuclear inclusions. The intranuclear inclusions were not membrane bound and were highly irregular in shape, but of the same electron density as those seen in the cytoplasmic organelles. Some of the intracytoplasmic inclusions appeared to be present in lysosomes in addition to the mitochondria, which might indicate degradation of inclusion-filled mitochondria with uptake into secondary lysosomes (Fig. 2E), often showing ultrastructural features similar to what is found in autophagosomes and autolysosomes. All cytoplasmic inclusions were within membrane-bound structures; no electron-dense inclusions were identified lying free within the cytoplasm. Similar findings were observed in enriched subcellular fractions from iAs\(^{\text{V}}\)-treated As3mt KO mice (Fig. 3). In the enriched subcellular fraction from WT mice treated with iAs\(^{\text{V}}\), inclusions were not present in nuclei. It was noted that female mice exhibited larger numbers and larger sized inclusions than male mice.

Quantitative Speciation of Arsenic Composition of Mouse Urothelial Inclusions

Chemical analysis of the enriched fractions of mitochondria and nuclei from mice treated with iAs\(^{\text{V}}\) showed that the majority of the arsenic is free and not bound to macromolecules.

iAs\(^{\text{V}}\), which was administered to the WT and As3mt KO mice, was the predominant form of unbound arsenical found in the mitochondrial and nuclear fractions from the WT mice and in the mitochondrial fraction from the As3mt KO mice (Table 1). iAs\(^{\text{III}}\) contributed to total arsenic content (18%) only in the mitochondrial fraction from treated As3mt KO mice. iAs\(^{\text{III}}\) was not detectable in this fraction from treated WT mice. DMA was detected in mitochondrial and nuclear fractions from both treated WT (8%) and treated As3mt KO mice (6%), but it contributed less to the total arsenic than did iAs. The As3mt null genotype appears to increase accumulation of iAs\(^{\text{III}}\) compared with other arsenic species.

In the enriched nuclear fraction from the treated WT group, iAs\(^{\text{V}}\) (54%) and DMA (38%) were the major contributors to the total arsenic measured. In contrast, iAs\(^{\text{III}}\) (59%) was the major arsenic species followed by iAs\(^{\text{V}}\) (28%) and DMA (12%) in the enriched fraction from treated As3mt KO mice.

TMAO was not detected either in the mitochondrial or in the nuclear fractions from any of the groups. Total arsenic concentration was highest in the nuclear fraction from the As3mt KO group due to the high concentration of iAs\(^{\text{III}}\).

Macromolecular-bound arsenic was detected only in the mitochondrial fraction from both WT (0.00053 nm) and As3mt (0.0019 nm) treated groups. The amount present in the nuclear fraction from both WT and As3mt mice was below the level of detection so that macromolecular binding of As could not be assessed. The amount of macromolecular-bound arsenic in the mitochondrial fraction was greater in the treated As3mt KO group than in the treated WT group, which correlated with the amount of iAs\(^{\text{III}}\) + DMA rather than with pentavalent iAs\(^{\text{V}}\).

FIG. 1. By H&E staining, eosinophilic intracellular inclusions were observed in the urothelium of mice administered iAs\(^{\text{V}}\). A, The urothelium of untreated (0 ppm As) mice. B, The presence of intracytoplasmic eosinophilic inclusions (arrows) of varying sizes in the superficial layer of the urothelium in WT C57BL/6 female mice administered 25 ppm iAs\(^{\text{V}}\) (104 ppm sodium arsenate) for 4 weeks. C, The presence of numerous intracytoplasmic (solid arrows) and intranuclear (dashed arrows) inclusions in all layers of the urothelium of As3mt KO female mice administered 25 ppm iAs\(^{\text{V}}\) for 4 weeks. ×1000. Abbreviations: As3mt KO, arsenic (+3 oxidation state) methyltransferase knockout; H&E, hematoxylin & eosin; iAs\(^{\text{V}}\), arsenate; WT, wild type.
Inclusion Formation in Untreated As3mt KO Mice

Male and female As3mt KO mice maintained in the breeding colony at UNMC were exposed only to the background iAs naturally present in the drinking water at levels < 8 ppb and in the diet at levels < 0.53 ppm (Teklad 8656). A few, small intracytoplasmic inclusions were observed in the urothelium of female As3mt KO mice at 165 and 439 days of age, detected by light microscopy. No inclusions were observed in male Asm3t KO mice at these ages by light microscopy, but intramitochondrial inclusions were detectable by TEM even at the age of 82 days in both male and female As3mt KO mice.

Inclusions in Urothelium Are Not Micronuclei

Inclusions in iAs^v^-treated WT and As3mt KO mice stained positive with Giemsa stain (Fig. 4I). However, the inclusions were negative when stained with DAPI (Figs. 4A–F; Table 2), indicating that the inclusions do not contain DNA. In combination with the electron microscopic appearance, the staining pattern indicates that the inclusions do not contain chromosomal material and are not micronuclei.

DISCUSSION

iAs is a human carcinogen (IARC, 2004; National Research Council, 2001). It is nonmutagenic (Hughes et al., 2011), and accumulating evidence supports a mode of action involving cytotoxicity and regenerative proliferation (Cohen et al., 2007, 2013). The presence of “micronuclei” in exfoliated urothelial cells from people exposed to high levels of iAs for long periods of time has been reported in multiple epidemiological studies (Basu et al., 2002; Ghosh et al., 2008; Moore et al., 1997) and has been suggested as evidence for an indirect genotoxic mechanism of iAs-induced carcinogenicity (Hughes et al., 2011). However, Suzuki et al. (2008a) reported the presence of intracytoplasmic eosinophilic inclusions (referred to as granules in earlier reports) in the urothelium of mice treated with arsenicals (iAs, DMA^v^), which were morphologically similar in appearance to those lesions claimed to be micronuclei. Recently, inclusions similar to those in mice were observed in exfoliated urothelial cells in urine from PML patients treated with ATO (150 µg/kg body weight/day for 30 days IV) (Wedel et al., 2013), and those ATO-associated inclusions appeared to be the same as those reported in epidemiology studies as micronuclei (Basu et al., 2002; Ghosh et al., 2008). In contrast, these inclusions were not observed in rats (Suzuki et al., 2008a), and rats do not produce micronuclei in response to arsenical exposure (Wang et al., 2009).

Similar inclusions in humans chronically exposed to high levels of arsenic through drinking water have been reported as...
"micronuclei" (Ghosh et al., 2008) using Giemsa (Basu et al., 2002), Feulgen (Gonsebatt et al., 1997; Tian et al., 2001), or propidium iodide (Moore et al., 1996) staining techniques. Feulgen staining is relatively more specific than Giemsa (Nersesyan et al., 2006). However, it has been shown that lead inclusions in human and rodent tissues give a false positive reaction with the
### TABLE 2
Summary of Characteristics of Intracellular Inclusions in Urothelium Induced by Exposure to Various Arsenicals in Mice and Human

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Treatment</th>
<th>Observed Time</th>
<th>Location in Urothelium</th>
<th>Characteristics of Inclusions</th>
<th>Staining Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>As3mt KO mice</td>
<td>Female</td>
<td>Regular drinking water and feed</td>
<td>Day 165 and day 439</td>
<td>Superficial layer</td>
<td>Cytoplasm: Yes; Nuclei: No</td>
<td>H&amp;E: Eosinophilic</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Regular drinking water and feed</td>
<td>2 weeks</td>
<td>All layers (iAs), Superficial (DMAIII)</td>
<td>Cytoplasm: Yes (mitochondria and lysosomes); Nuclei: Yes (with iAs)</td>
<td>H&amp;E: Eosinophilic, Giemsa: Yes, DAPI: No</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>C57BL/6 WT mice</td>
<td>Female</td>
<td>iAsV/iAsIII/DMA III</td>
<td>7 days, 14 days, 2 weeks</td>
<td>Superficial layer</td>
<td>Cytoplasm: Yes; Nuclei: No</td>
<td>H&amp;E: Eosinophilic</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td>2 weeks</td>
<td>All layers (iAs), Superficial (DMAIII)</td>
<td>Cytoplasm: Yes (mitochondria and lysosomes); Nuclei: Yes (with iAs)</td>
<td>H&amp;E: Eosinophilic, Giemsa: Yes, DAPI: No</td>
</tr>
<tr>
<td></td>
<td>Male and Female</td>
<td>iAsV, iAsIII/DMA III</td>
<td>2 or 10 weeks 2 years</td>
<td>Superficial layer</td>
<td>Cytoplasm: Yes; Nuclei: No</td>
<td>H&amp;E: Eosinophilic</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>High levels of iAs in drinking water</td>
<td>Chronic exposure 0, 3, and 7 months after stopping treatment</td>
<td>Superficial layer</td>
<td>Cytoplasm: Yes; Nuclei: No</td>
<td>H&amp;E: Yes (data not shown), Basophilic: No</td>
</tr>
</tbody>
</table>

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*Suzuki et al. (2008a).*
*Yokohira et al. (2011).*
*Yokohira et al. (2010).*
*Dodmane et al. (2013).*
*Suzuki et al. (2008b).*
*Arnold et al. (2006).*
*Basu et al. (2002).*
*Wedel et al. (2013).*
Feulgen stain (Beaver, 1961; Wachstein, 1949; Wolman, 1954), and therefore, they may also give a false positive when staining arsenic-induced metal ion-rich inclusions. Propidium iodide is an intercalating agent that binds DNA and RNA. In addition to its high affinity for nuclei acids (Tas and Westerneng, 1981), propidium is an inhibitor of acetylcholine esterase by binding strongly to its peripheral anionic site (Cavalli et al., 2004). This suggests that propidium iodide can bind to other molecules in the cell, apart from nucleic acids. We recently reported (Wedel et al., 2013) the presence of similar inclusions in exfoliated urothelial cells collected from the urine of ATO-treated PML patients. These inclusions showed negative staining for DAPI, a DNA-specific stain, and by TEM, the inclusions did not resemble micronuclei. Similarly, the inclusions in the mouse urothelial cells do not stain with DAPI and do not resemble nuclei by TEM. The intracytoplasmic inclusions are contained in membrane-bound organelles, which are not free in the cytoplasm. In studies with arsenic exposure where these “micronuclei” were categorized based on the absence or presence of centromere staining, there was an increase in centromere negative “micronuclei” compared with centromere positive “micronuclei” (Moore et al., 1997). Rather than being micronuclei, they likely represent the arsenic-rich inclusions we are describing. In a few other studies, using the same method the prevalence of micronuclei was not significant compared with the unexposed group although a trend was associated with arsenic intake (Moore et al., 1996; Warner et al., 1994). These findings suggest that the inclusions are not micronuclei in either mice or humans, and they do not indicate genotoxicity of arsenicals.

The arsenicals iAsV, iAsIII, and DMAIII all induced formation of these inclusions in the mouse urothelium in both WT (C57BL/6) and As3mt KO mice (Arnold et al., 2013; Simeonova et al., 2001; Suzuki et al., 2008a; Yokohira et al., 2010, 2011). DMAV-induced inclusions were observed in B6C3F1 mice when administered through the feed (Arnold et al., 2006). Inclusions in urothelial cells were also observed in the bladders of B6C3F1 mice administered iAsIII in the drinking water in studies conducted by the National Toxicology Program (Dr Robert Maranpot, personal communication), which were similar in appearance to what we have observed (Suzuki et al., 2008a). This suggests that the induction of inclusions is not limited to one strain of mice nor is it limited to one form of administered arsenical. Based on previous studies (Arnold et al., 2013; Dodmane et al., 2013; Suzuki et al., 2008a; Yokohira et al., 2010, 2011) and the present studies, the inclusions appear similar when stained with H&E and are produced whether the arsenical is administered in the diet or in the drinking water.

In the WT mice, the inclusions were confined to the cytoplasm in the superficial cell layer of the urothelium. In As3mt KO mice, the inclusions were also present in the nuclei and in all layers of the urothelium, which is likely due to slow clearance of iAs because of lack of methylation ability (Drobna et al., 2009). The presence of the inclusions in untreated mice likely corresponds to the poor ability of these As3mt KO mice to eliminate iAs (Drobna et al., 2009).

Chemical analysis showed that in iAsV-treated mice, WT or KO, the major arsenical in the enriched mitochondrial fractions was unbound iAsV, whereas in As3mt KO mice, the major arsenical in the nuclear fraction was iAsIII. In a previous study, iAsIII was found to be the predominant arsenic species in the enriched mitochondrial fraction from WT mice administered iAsIII (Suzuki et al., 2008a). Therefore, it appears that the major form of the arsenical present in the mitochondrial inclusions depends on the form of arsenical administered to the mice. Unlike in urine, where DMAV is the major metabolite in WT mice, iAs (iAsIII + iAsV) was the major arsenical in mitochondrial and nuclear fractions. This finding is in agreement with findings in human exfoliated urothelial cells from people exposed to high levels of iAs in the drinking water (Hernández-Zavala et al., 2008) and also with our earlier report in mice treated with iAsIII (Suzuki et al., 2008a). The arsenic species in urine do not directly correspond quantitatively to the arsenical species in the urothelial cells in mice or humans. Compared with the WT mice, As3mt KO mice appeared to store more arsenic with iAsV treatment in both mitochondria and nuclei, mainly in the form of iAsIII, consistent with disrupted kinetics of iAs in As3mt KO mice (Drobna et al., 2009).

In this study, the majority of the arsenic found in the enriched organelles was free and not bound to macromolecules. The small amount of macromolecule-bound arsenic in the enriched mitochondrial fractions coincided with amounts of AsIII + DMA but not with iAsV. This difference might be due to higher binding affinity of the trivalent iAsIII and DMAIII compared with the pentavalent iAsV (Jiang et al., 2003; Lu et al., 2004, 2007). Unlike the mitochondrial fraction, macromolecule-bound arsenic was not detected in the enriched nuclear fractions. This might be due to weak binding of arsenic to a macromolecule or a difference in the storage buffer used for the enriched mitochondrial and nuclear fractions.

Formation of these inclusions in mice is dose (Supplementary Table S1) and time dependent (Arnold et al., 2013). In As3mt KO mice, intracytoplasmic inclusions were observed as early as 6 h postexposure to drinking water containing 25 ppm of iAsIII compared with 7 days in the WT mice. In both WT and As3mt KO mice, exposure to iAsIII in drinking water for 4 weeks induced inclusions depending on dose; 1 ppm iAsIII induced fewer inclusions than 10 ppm or 25 ppm iAsIII. The location of the inclusions was also dose dependent in the As3mt KO mice, with intracytoplasmic inclusions present in the superficial layer of the urothelium at low doses (< 10 ppm) but in all layers of the urothelium and in the nuclei at higher doses (≥ 10 ppm). Even the background arsenic in the regular drinking water (< 8 ppb) produced a few inclusions in the breeding colony of As3mt KO mice; they were small and were found only in the superficial cells. Again, this is consistent with the kinetics for iAs in these KO mice (Drobna et al., 2009).
The significance of these inclusions for the biological effects of arsenic in mice or in humans is unclear. However, the presence of the inclusions does not appear to be involved in the arsenical-induced cytotoxicity or hyperplasia of the urothelium in mice, as they were present in the urothelium of mice treated with iAs that did not show toxic effects (Suzuki et al., 2008a; Yokohira et al., 2010, 2011), and they were not found in rats, which show similar cytotoxic and proliferative effects to iAs exposure as mice (Arnold et al., 2013). Furthermore, they were present in mice treated with DMA\textsuperscript{V} in the diet, even though that exposure did not produce cytotoxic, proliferative, or tumor effects in the mice, but they were not present in rats in which cytotoxic, proliferative, and tumor effects were induced by DMA\textsuperscript{V} fed in the diet (Arnold et al., 2006). This suggests that these inclusions may act as a depot to sequester arsenicals and prevent adverse effects on cells.

Other metal ions, such as lead and cadmium, have been observed to induce intracytoplasmic and intranuclear inclusions or aggregates (Gonick, 2011; Moore and Goyer, 1974; Song et al., 2008; Wachstein, 1949). As with arsenicals, such inclusions associated with other metals appear to serve to sequester the metal and do not represent a toxic effect of the metal. Many of these metal ions, including inorganic and organic arsenicals, are known to bind to metallothionein (Park et al., 2001). In addition, lead has been shown to bind to specific proteins in the inclusions (Gonick, 2011). Lipofuscin was shown by light microscopy to form inclusions in the urothelial superficial layer of mice exposed to stress (Perše et al., 2013). However, the inclusions induced by arsenicals were negative for immunohistochemical labeling of metallothionein, and they were negative for Fontana Masson staining, a stain for lipofuscin (data not shown). The specific macromolecules to which the arsenicals are bound in these inclusions remain unknown.

The urothelium has a very slow turnover rate in most species, from 6 months to 2 years (Jost, 1989; Khandelwal et al., 2009; Rebel et al., 1994; Tiltman and Friedell, 1972), unless there is cytotoxicity to the urothelium. The inclusions persisted even 7 months after the end of the arsenic treatment in human PML patients (Wedel et al., 2013). We have also found that a few inclusions remain in the mice 3 months after cessation of the iAs\textsuperscript{IV} treatment. Such persistence is consistent with the slow loss by exfoliation over time with the normal turnover of the urothelium and is consistent with the inclusions being noncytotoxic. If cytotoxic, a more rapid turnover would be expected (Cohen, 1998).

In summary, exposure to iAs induced formation of inclusions in the mouse urothelium that appear similar to the inclusions found in exfoliated urothelial cells from humans exposed to high levels of iAs. The number, extent of urothelial involvement, and time of appearance are dose dependent. The inclusions appear to serve as a depot for arsenicals and are not related to arsenical-induced urothelial toxicity. In humans, these inclusions have been mistaken for micronuclei. The current findings show that these inclusions are not micronuclei and that they are not evidence of genotoxicity.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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