Urothelial Cell Intracytoplasmic Inclusions After Treatment of Promyelocytic Leukemia With Arsenic Trioxide

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Intramitochondrial inclusions containing arsenite that occur within urothelial cells have been previously described in mice exposed to high concentrations of arsenic but not in rats. In epidemiology studies, similar urothelial cell inclusions have also been observed in the urine of humans exposed to high concentrations of arsenic in the drinking water; however, these inclusions were mistakenly identified as micronuclei. To further examine the urothelial cell inclusions that occur in inorganic arsenic-exposed humans, we evaluated two patients with a history of acute promyelocytic leukemia treated for disease relapse with a combination of all-trans retinoic acid and arsenic trioxide. Posttreatment examination of the patients’ urine cytology specimens by light and electron microscopy demonstrated cytoplasmic inclusions in exfoliated superficial urothelial cells similar to those seen in mice. The inclusions were present in decreasing quantities at 3 and 7 months after completion of treatment. No comparable inclusions were detected in exfoliated urothelial cells in urine from six individuals not treated with arsenic trioxide. Based on the results of the examination by light and electron microscopy, we have determined that urothelial cell inclusions in the urine of humans previously identified as micronuclei are instead intracytoplasmic inclusions similar to those found in arsenic-treated mice.

Key Words: urothelial cell; arsenic trioxide, bladder.

Inorganic arsenic (arsenite or arsenate) is a known human carcinogen for the urinary bladder, lung, and skin (NRC, 1999, 2001). In mouse models, intracytoplasmic inclusions have been observed within the urothelium of mice orally administered high doses of arsenite or arsenate. These inclusions were shown to be intramitochondrial by electron microscopy and contain arsenite bound to protein (Suzuki et al., 2008). The inclusions do not appear to be necessary for the arsenic-induced cytotoxicity and regenerative hyperplasia that occur in the urothelium of treated mice because they do not occur in arsenic-treated rats that show similar cytotoxicity and regeneration. Instead, they may be a possible protective mechanism as has been seen with other metals such as lead (Brown et al., 1985; Gonick, 2011; Masci et al., 1995).

In humans, epidemiologic studies evaluating arsenic-related urothelial cytologic effects have reported the presence of cytoplasmic inclusions in exfoliated human urothelial cells in the urine of individuals exposed to high levels of arsenic in the drinking water (Ghosh et al., 2008; Moore et al., 1997). These inclusions have been classified as micronuclei. However, in some studies, micronuclei were identified based on positive staining with the nonspecific Giemsa stain, which has been shown to give false positive results in epithelial cells (Nersesyan et al., 2006). In studies using centromere-specific stains, often the majority of inclusions identified as micronuclei were centromere negative (Ghosh et al., 2008; Marchiset-Ferlay et al., 2012; Moore et al., 1997).

To evaluate the possibility that these inclusions occurring in arsenic-exposed humans are not micronuclei, but rather similar to the arsenic-containing granules observed in mice, we evaluated two patients with acute promyelocytic leukemia (APL) treated with arsenic-based chemotherapy. Both patients had intracytoplasmic inclusions in exfoliated urothelial cells present in their urine similar to those seen in the urothelium of mice. By light microscopy these inclusions were indistinguishable from what has been classified as micronuclei in epidemiologic studies of populations exposed to high concentrations of inorganic arsenic in the drinking water, but by electron microscopy we show that they are the same intracytoplasmic inclusions detected in mice and distinct from micronuclei. In addition, these same inclusions did not stain with the DNA-specific stain 4′,6-diamidino-2-phenylindole (DAPI).
MATERIALS AND METHODS

Chemical
Arsenic trioxide (ATO) (Cephalon Inc., Frazer, PA) was obtained from the Nebraska Medical Center inpatient pharmacy and administered iv at a dose of 0.15 mg/kg body weight per day to the APL patients. The study protocol was approved by the University of Nebraska Medical Center Institutional Review Board.

Control Subjects
Six healthy female volunteers, aged 25–69 years, were recruited from the student and employee population at the University of Nebraska Medical Center (UNMC) and the Nebraska Medical Center.

ATO-Treated Subjects
Case 1. The first patient is a 36-year-old female with a history of APL, which was first diagnosed at age 30. Initial cytogenetic analysis showed the classic t(15;17)(q22;q11.2) translocation. The patient entered complete remission after treatment with all-trans retinoic acid (ATRA) and daunorubicin, followed by consolidation therapy with cytarabine and ATO. ATO was administered iv at a dose of 0.15 mg/kg for 30 consecutive days. However, 6 years after entering remission, the patient was found to have leukemia and thrombocytopenia, and a bone marrow biopsy showed disease relapse with hypercellular bone marrow (80%) composed of 53% blasts and promyelocytes. Concurrent flow cytometry completed on the bone marrow specimen was consistent with relapsed APL. The patient was subsequently treated with two courses of ATO (0.15 mg/kg, iv) for 30 consecutive days, each time with 3 weeks between the two courses. After treatment, the patient again reached complete remission and subsequently underwent autologous stem cell transplantation.

Case 2. The second patient is a 43-year-old male with a history of APL diagnosed at age 38. Initial cytogenetic studies revealed the presence of an abnormal diploid clone, which was characterized by a reciprocal translocation involving 15q and 17q, and two subclones; the first subclone was characterized by an interstitial deletion of 5q, and the second subclone was characterized by an interstitial deletion of 5q and additional unknown material on 7q. The patient received induction chemotherapy composed of anthracycline and ATRA, with consolidation therapy using idarubicin and cytarabine. Subsequently, the patient received maintenance therapy with ATRA, methotrexate, and 6-mercaptopurine for 2 years. On this regimen, the patient entered complete morphologic and molecular remission. However, 5 years after initial diagnosis, the patient was noted to have mild thrombocytopenia (platelet count: 140,000/µl [ref: 150,000–400,000/µl]) and underwent bone marrow biopsy, which showed disease relapse with normocellular bone marrow containing 8% blasts. Cytogenetic analysis showed identical material on 7q. The patient received induction chemotherapy composed of cytarabine, followed by consolidative therapy with cytarabine and ATO. ATO was administered iv at a dose of 0.15 mg/kg, iv) for 30 consecutive days, each time with 3 weeks between the two courses. After treatment, the patient again reached complete remission and subsequently underwent autologous stem cell transplantation.

Urine Samples
Control subjects. Random urines collected at various times during the day were collected from six control subjects. Samples were processed for examination by light and electron microscopy.

ATO-treated subjects. In the first case, random afternoon fresh void urine was collected after completion of 30 days of ATO treatment and again 3 and 7 months after termination of treatment. In the second case, a random afternoon fresh void urine specimen was collected after completion of 60 days of ATO treatment. Samples for both cases were processed for examination by light microscopy and transmission electron microscopy and, in the first case, by fluorescent microscopy.

Light Microscopy
One half of each urine sample was fixed in an equal volume of Shandon Cytospin Collection Fluid (Thermo Scientific, St Louis, MO). The preserved sample was stored at approximately 4°C until it was transported to the cytology laboratory for processing. Specimens were spun at 2000 rpm for 10 min, the supernatant was removed, and the cell button was resuspended in saline (two cycles). Then, three to four drops of the solution were placed into a plastic chamber holding a glass slide; the glass slide (in holder with solution) was spun at 2000 rpm for 5 min. The glass slide was then removed from the plastic chamber and stained with hematoxylin and eosin (H & E).

Fluorescent Microscopy
After examination by light microscopy, the H&E-stained slide from the urine sample collected in the first case after 30 days treatment with ATO was scanned and coordinates and images of cells containing inclusions were captured. The slide was destained to remove most of the H&E stain, restained with DAPI, and scanned using fluorescent microscopy. Based on the coordinates in the two scanned images, cells containing inclusions were identified.

Transmission Electron Microscopy
The remaining half of each urine sample was spun for 10 min at 1700 rpm, and the supernatant was removed. The remaining sediment was fixed in 5–10 ml EM fixative, (Millonig’s Fixative (MPG), 2.0% paraformaldehyde/2.5% gluteraldehyde) and stored at approximately 4°C until it was transported to the EM laboratory for processing. Specimens were spun at 1700 rpm for 10 min in a plastic conical centrifuge tube to form a loose pellet of cells. Supernatant was removed and replaced with 300 ml of histogel (Thermo Scientific) at 65°C and allowed to cool to room temperature. The cell pellet was removed, dissected into 1-mm cubes, and postfixed in EM fixative at 4°C for 1 h. EM blocks were processed on an automated EM tissue processor (Leica EM TP, Buffalo Grove, IL). The blocks underwent secondary fixation with osmium tetroxide, after which they were washed in three additional deionized water for 10 min (x2). The tissue was then dehydrated with a series of ethyl alcohol dilutions, followed by treatment with acetone/tolirn (three changes for 5 min). The tissue was then infiltrated with fresh resin according to the following infiltration methods: 1:1 acetone/tolirn:resin for 1 h; fresh Poly/Bed 812 resin for 1 h. Tissue was removed from the processor and then placed in fresh Poly/Bed 812 resin to polymerize in an oven at 60°C for 48 h. Plastic survey thick sections were obtained at 1 µm, stained with 0.5% toluidine blue, and examined under the light microscope. Selected areas were ultrasectioned at 70–90 nm (silver sections), mounted on copper grids and stained with uranyl acetate and lead citrate, and examined in the electron microscope (JEOL 1230, Peabody, MA).

RESULTS

Light Microscopic Examination
Control subjects. No inclusions were observed in the urothelial cells present in the urine of control subjects when examined by light microscopy.

ATO-treated subjects. By light microscopy, in both cases, round, eosinophilic-to-deep blue intracytoplasmic inclusions were detected in most exfoliated superficial urothelial cells in the specimens collected at the end of treatment, often with many inclusions of various sizes present in individual cells. There was no evidence of cellular necrosis or apoptosis (Fig. 1A). In case 1, in urine samples collected approximately 3 and 7 months after ATO treatment was stopped, the intracytoplasmic inclusions were still present in the exfoliated superficial urothelial cells but were fewer in numbers in individual cells,
and inclusions were not present in all of the cells (Fig. 1B). In the 7-month posttreatment urine specimen, only 10–20% of the exfoliated urothelial cells contained inclusions.

Fluorescent Microscopy

Intracytoplasmic inclusions present in the exfoliated urothelial cells in urine collected after 30 days treatment with ATO in case one did not stain with the DNA-specific stain DAPI (Fig. 2).

Transmission Electron Microscopy Examination

Control subjects. No abnormalities were observed by TEM in the urines from control subjects.

ATO-treated subjects. Investigation by TEM of cell pellets from all time points in case 1 and from case 2 showed that the intracytoplasmic inclusions were round, present in membrane-bound round organelles, and varied in size. Although we were not able to definitively ascertain in which organelle the granules were present, they appeared to be present in lysosomes (Fig. 3).

DISCUSSION

Pharmacologic use of arsenic-based chemotherapy was common in the mid-1800s to the early 1900s but fell out of favor with the advent of less-toxic therapies (Kwong and Todd, 1997). More recent studies have shown the efficacy of treatment using ATO treatment in patients with APL, reviving its medical use (Chen et al., 1997; Shen et al., 1997). However, exposure to high levels of environmental inorganic arsenic is a known cause of multiple cancers in humans, including urothelial carcinoma (Smith et al., 1992; Wu et al., 1989).

Exposure to high concentrations of other heavy metals, such as lead, results in their concentration within mitochondria (Brown et al., 1985; Gonick, 2011). Our prior studies investigating the mechanism of arsenic-induced urothelial cytotoxicity using rat and mouse models demonstrated intramitochondrial inclusions containing inorganic arsenic within the urothelium of mice treated with high levels of inorganic or organic arsenic; the same inclusions were not seen in the urothelium of similarly treated rats (Suzuki et al., 2008). The major form of arsenic found in the mouse inclusions was inorganic arsenite similar to the results Hernández-Zavala et al. (2008) found when they analyzed the arsenic content of exfoliated urothelial cells in the urine of humans exposed to arsenic in the drinking water (< 1 to 190 µg arsenic/l).

We observed inclusions similar to those in mice by light and transmission microscopy in exfoliated human urothelial cells in the urine of two patients treated with ATO. The inclusions were not associated with signs of necrosis or apoptosis, suggesting that they represent a cellular depot collection of bound arsenite not associated with toxicity. This conclusion is further supported by their continued presence 3 and 7 months after cessation of treatment. The urothelium is a slowly proliferating tissue in most species with turnover times estimated at 6 months to 2 years (Rebel et al., 1994; Tiltman and Friedell, 1972). It appears that the inclusions remain until the superficial cells are exfoliated as part of the normal turnover of the urothelium. Therefore, the presence of these inclusions in exfoliated
urothelial cells in the urine provides a marker of high exposure to inorganic arsenic, whether from environmental exposure or from arsenic-containing pharmaceuticals in a specimen which can be obtained by noninvasive techniques.

In mice, the urothelial inclusions occur in the mitochondria (Suzuki et al., 2008). In mouse studies, the urothelium is well preserved as the bladder is inflated with fixative while the animal is under deep anesthesia, preventing autolysis (Suzuki et al., 2008). Under such circumstances, the organelle structures are well preserved, making it possible to identify the presence of the inclusions in mitochondria. In the present studies in humans, the cells are exfoliated superficial cells present in urine and have degenerative changes associated with autolysis. Nevertheless, the overall impression by electron microscopy is that the inclusions are present in lysosomes in these human specimens, in contrast to their location in mitochondria in mice. However, the precise location of these inclusions in the human cells requires further investigation.

Previous studies have shown an increased incidence of cytoplasmic inclusions classified as micronuclei in exfoliated bladder cells from individuals exposed to high levels of inorganic arsenic in the drinking water, suggesting possible genotoxicity (Ghosh et al., 2008). In some studies, the apparent micronuclei were detected using the Giemsa stain, a nonspecific stain which has been shown to increase false positive identification of micronuclei (Nersesyan et al., 2006). In this study, we
showed that these cytoplasmic inclusions do not stain when the DNA-specific stain DAPI is used. The intracytoplasmic inclusions observed in the urothelial cells of arsenic-treated mice also do not stain with DAPI (unpublished observations). In other studies, centromere markers were used, but most of the increase in apparent micronuclei were in centromere-negative micronuclei (Ghosh et al., 2008; Marchiset-Ferlay et al., 2012; Moore et al., 1997). Some micronuclei are present in urothelial cells normally as in most cells (Nersesyan et al., 2006). Because the micronuclei and cytoplasmic arsenic inclusions have an identical appearance by light microscopy, it is likely that most if not all of these centromere-negative inclusions are actually inclusions composed of arsenic. The appearance by electron microscopy of the inclusions in the cases we examined show that they are contained in membrane-bound organelles and do not have the appearance of micronuclei.

In conclusion, based on the results of the examination by light, fluorescent, and electron microscopy, we have determined that urothelial cell inclusions in the urine of humans exposed to high levels of arsenic previously identified as micronuclei are instead intracytoplasmic inclusions similar to those found in arsenic-treated mice.

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REFERENCES


