Urothelial Cells Malignantly Transformed by Exposure to Cadmium (Cd$^{+2}$) and Arsenite (As$^{+3}$) Have Increased Resistance to Cd$^{+2}$ and As$^{+3}$-Induced Cell Death

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This laboratory has shown that both Cd$^{+2}$ and As$^{+3}$ can malignantly transform human urothelial cells. The present study examined metal resistance and the mechanism of cell death when the parental and malignantly transformed UROtsa cells were exposed to Cd$^{+2}$ and As$^{+3}$. It was shown that the malignantly transformed UROtsa cells were more resistant to the toxic effects of both metals. The assessment of the mode of cell death demonstrated that the parental UROtsa cells died by both apoptosis and necrosis when exposed to either metal. It was shown that apoptosis was the more prominent mechanism of cell death, accounting for over 50% of cell death. Apoptotic cell death was determined by the observation of fragmented nuclei using 4',6-diamidino-2-phenylindole staining, the formation of a DNA ladder, and the detection of cleaved caspase-3 and caspase-9 products in the cell lysates. Necrotic cell death was determined by measuring the release of lactate dehydrogenase into the growth medium. It was determined that the extent of apoptosis of the malignantly transformed UROtsa cells was decreased and that the extent of necrosis was increased compared to the parental UROtsa cells. These observations are consistent with in vivo studies which suggest that As$^{+3}$ can act as a tumor promoter during the regeneration of the bladder urothelium. The present in vitro studies suggest that As$^{+3}$-induced cytotoxicity could set the stage for tissue repair due to its own inherent toxicity to normal urothelium, and then subsequently act as a tumor promoter during the regeneration process through the stimulation of the regrowth of cells that have gained increased resistance to As$^{+3}$.

Key Words: arsenite; cadmium; urothelium; bladder cancer; apoptosis; necrosis.

Transitional cell carcinoma of the bladder is the fourth most common cancer in men and the fifth in women in Western countries (Johansson and Cohen, 1997). Historically, bladder cancer is the first cancer in which environmental agents were found to play the major role in disease causation. This was first observed by Rehn in 1895 when a link was demonstrated between exposure to aromatic amines and development of bladder cancer in factory workers (Rehn, 1895). This association was subsequently confirmed in both animal models and humans working in industries that involve exposure to aromatic amines (Case et al., 1954; Hueper et al., 1938). In more recent times, an association between cigarette smoking and bladder cancer was found, with some reports suggesting a twofold increased risk and that 50% of the bladder cancers in men would not occur in the absence of cigarette smoking (Clavel et al., 1989; Morrison et al., 1984). Most recently, epidemiologic evidence has provided substantial evidence linking the ingestion of arsenic in drinking water with the development of bladder cancer (Steinmaus et al., 2000). Epidemiologic studies have shown a strong association between arsenic ingestion from contaminated drinking water and the development of bladder cancer in Taiwan (Chiou et al., 1995), Argentina (Hopenhayn-Rich et al., 1996), Chile (Smith et al., 1998), and Japan (Tsuda et al., 1995). Arsenic is classified by the International Agency for Research on Cancer as a human carcinogen (IARC, 1980), and it is presently first in priority among a listing of the top 20 hazardous substances by the Agency for Toxic Substances and Disease Registry and the U.S. Environmental Protection Agency (ATSDR, 1997). Another heavy metal, cadmium (Cd$^{+2}$), is also a known human carcinogen that has been associated with the development of bladder cancer (Siemiatycki et al., 1994; Waalkes, 2000), although the epidemiologic data is much less extensive. Like arsenic, cadmium has been classified by the International Agency for Research on Cancer as a human carcinogen (IARC, 1993).

There are limited human models of bladder cancer available for study where malignant transformation has been induced by environmental agents. This laboratory has recently directly malignantly transformed an immortalized cell culture of human urothelial cells (UROtsa) with both Cd$^{+2}$ and As$^{+3}$ (Sens et al., 2004). This required extended exposure of the cell line to 1μM Cd$^{+2}$ or As$^{+3}$. The resulting transformed cells were shown to have increased growth rates and to readily form colonies in soft agar when compared to untreated control cells. The transformed cells arising from treatment with both Cd$^{+2}$ and As$^{+3}$ were
shown to form tumors when injected sc into immunocompromised (nude) mice. The histology of the tumor heterotransplants displayed the features expected of human transitional cell carcinoma of the bladder. An interesting observation in this study was that before the resulting cell cultures gained the ability to form colonies in soft agar, and subsequently tumors in nude mice, both cell lines experienced two similar episodes of As\(^{3-}\)_2- and Cd\(^{2+}\)-induced cell death and subsequent regrowth of the culture to confluency. This may also occur within the human bladder under conditions of metal exposure where the urothelium undergoes cell death followed by proliferation to a complete transitional epithelium. The role of cell proliferation preceded by toxicant-induced cell death has been intimately associated with the models of carcinogenesis particularly in the liver (Oliver and Roberts, 2002) and the cancer-causing effect of one arsenic compound, dimethylarsenic acid (DMA) may be related to the observed cycle of cell death and regeneration (Cohen et al., 2001). 

The observed cycle of proliferation preceded by metal-induced cell death during the UROtsa in vitro transformation process raised several questions. First, does the transformation process alter the susceptibility of the transformed cells to further exposure to Cd\(^{2+}\) and As\(^{3-}\), thereby enhancing the accumulation of transformed cells within the urothelium? Second, what is the mechanism of cell death in human urothelial cells exposed to As\(^{3-}\) and Cd\(^{2+}\), does the transformation process alter the mechanism of cell death in human urothelial cells and is it the same for both metals? Lastly, does the intracellular level of the metal-binding protein, metallothionein (MT), influence the malignant transformation of urothelial cells exposed to Cd\(^{2+}\) or As\(^{3-}\)? The present study addresses these questions.

**MATERIALS AND METHODS**

**Cell culture.** Stock cultures of the parent UROtsa cells and UROtsa cell lines malignantly transformed with either 1μM Cd\(^{2+}\) or As\(^{3-}\) were maintained in 75 cm\(^2\) tissue culture flasks in Dulbecco’s modified Eagle’s medium containing 5% vol/vol fetal calf serum as described previously (Rossi et al., 2001; Sens et al., 2004). Cultures were incubated at 37°C in a 5% CO\(_2\): 95% air atmosphere. The cells were fed fresh growth medium every 3 days, and when confluent, the parental UROtsa cells were subcultured at a 1:4 ratio and the transformed cells at a 1:20 ratio using trypsin-EDTA. The Cd\(^{2+}\) and As\(^{3-}\)-transformed cell lines were serially passaged 10 times in Cd\(^{2+}\)- and As\(^{3-}\)-free growth medium before use in any experimental protocols. 

The parental cell line is defined by the designation UROtsa, cells transformed with Cd\(^{2+}\) on serum-containing growth medium by the designation URO-CDS, and cells transformed with As\(^{3-}\) on serum-containing growth medium by the designation URO-ASSC.

**Visualization of DAPI-stained cells.** The effect of metal treatment on cell viability and the number of fragmented (apoptotic) nuclei was visualized microscopically using 4’,6-diamidino-2-phenylindole (DAPI)-stained nuclei as described previously by this laboratory (Somji et al., 2004). At the indicated time points, wells containing the monolayers were rinsed with phosphate-buffered saline (PBS), fixed for 15 min in 70% ethanol, rehydrated with 1 ml PBS, and stained with 10 μl DAPI (10 μg/ml in distilled water).

**MTT assay for cell viability.** Cell viability, as a measure of cytotoxicity, was determined by measuring the capacity of the cells to reduce MTT (3-(4, 5-}

<ref>https://academic.oup.com/toxsci/article-abstract/94/2/293/1647370/293</ref>
measuring cell viability. For this purpose, confluent cultures of parent and transformed UROtsa cells were exposed to increasing concentrations of Cd\(^{2+}\) or As\(^{3+}\) for 24–48 h and cell viability was determined using the MTT assay. The results of this determination showed that Cd\(^{2+}\)-transformed cells were more resistant to the cytotoxic effects of Cd\(^{2+}\) compared to the parental cells (Figs. 2A–2C). Exposure of the URO-CDSC cells to the highest level of Cd\(^{2+}\) utilized in the study (27\(\mu\)M) for 48 h resulted in only a 50% loss in cell viability compared to the exposed parental cells. The URO-ASSC cells were modestly more resistant to As\(^{3+}\)-induced cell death than the parental UROtsa cells (Figs. 2D–2F). However, by 36 h, both the parental as well as the As\(^{3+}\)-transformed cells were sensitive to the cytotoxic effects of As\(^{3+}\).

**Apoptosis and Necrosis of UROtsa Cells Exposed to Cadmium**

One of the goals of this study was to determine if Cd\(^{2+}\) elicited apoptosis in UROtsa cells. The concentration range and time course of cadmium exposure for the determination of cadmium-induced apoptosis and necrosis was determined from the viability assays. This is important since the sensitivity of the assays (DNA laddering and caspase activation) used to confirm apoptosis require a significant number of apoptotic cells. The initial step was to determine if apoptosis did occur in parental UROtsa cells exposed to Cd\(^{2+}\). Evidence that apoptosis was active in Cd\(^{2+}\)-exposed UROtsa cells was obtained by observing triplicate cultures of confluent UROtsa cells exposed to various concentrations of Cd\(^{2+}\) over time and staining the cells with DAPI when a majority of the cell population acquired a “rounded” morphology. The fluorescent staining of nuclei using DAPI has been used previously by this laboratory to microscopically visualize the fragmented and condensed nuclei that are characteristic of apoptotic cells (Somji et al., 2004). This determination showed that 17.5 ± 1.4% of the UROtsa cells had condensed and fragmented nuclei when they were exposed to 16\(\mu\)M Cd\(^{2+}\) for 36 h (Fig. 1A). It was shown that exposure of parental UROtsa cells to 27\(\mu\)M Cd\(^{2+}\) resulted in the formation of a DNA ladder (Fig. 3A) and cleavage of caspases 3 and 9 (Fig. 4B). An analysis of LDH release into the growth medium showed a maximum release of 30% of total LDH from the cells under conditions of As\(^{3+}\) exposure that resulted in a total loss of cell viability (Fig. 5B).

**Apoptosis and Necrosis of Cd\(^{2+}\)-Transformed UROtsa (URO-CDSC) Cells Exposed to Cd\(^{2+}\)**

Another goal of this study was to determine if UROtsa cells malignantly transformed through exposure to Cd\(^{2+}\) would have an alteration in the mechanism of Cd\(^{2+}\)-induced cell death compared to that of the parental cells exposed to Cd\(^{2+}\). It was first determined using DAPI staining as described above to determine if putative apoptotic cell profiles could be found in URO-CDSC cells exposed to Cd\(^{2+}\). It was shown that 12 ± 1.2% of URO-CDSC cells exhibited profiles of condensed and fragmented nuclei when exposed to 20\(\mu\)M Cd\(^{2+}\) for 36 h, features consistent with cells undergoing apoptosis (Fig. 1C). DNA laddering and caspase activation could be shown for the URO-CDSC cells exposed to 27\(\mu\)M Cd\(^{2+}\) (Figs. 3C and 4C), exposure of parental UROtsa cells to 16\(\mu\)M As\(^{3+}\) resulted in the formation of a DNA ladder (Fig. 3B) and cleavage of caspases 3 and 9 (Fig. 4B). An analysis of LDH release into the growth medium showed a maximum release of 30% of total LDH from the cells under conditions of As\(^{3+}\) exposure that resulted in a total loss of cell viability (Fig. 5B).

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but in both instances the qualitative amounts of both were reduced compared to parental cells (Figs. 3A and 4A). This reduction was especially pronounced for the cleavage of caspase-3. An analysis of LDH release into the growth medium showed a release of 40–60% of total LDH from the cells under conditions of Cd\(^{+2}\) exposure that resulted in only a 50–60% loss of cell viability (Fig. 5C), a significant increase compared to the release of LDH by the parental cells (Fig. 5A).

**Apoptosis and Necrosis of As\(^{+3}\)-Transformed UROtsa (URO-ASSC) Cells Exposed to As\(^{+3}\)**

The next goal of this study was to determine if UROtsa cells malignantly transformed through exposure to As\(^{+3}\) would have an alteration in the mechanism of As\(^{+3}\)-induced cell death compared to that of the parental cells exposed to As\(^{+3}\). It was shown using DAPI staining that 9.6 ± 1.0% of URO-ASSC cells exhibited profiles of condensed and fragmented nuclei when exposed to 12\(\mu\)M As\(^{+3}\) for 30 h, features consistent with cells undergoing apoptosis (Fig. 1D). DNA laddering and caspase activation could be shown for the URO-ASSC cells exposed to 16\(\mu\)M As\(^{+3}\) (Figs. 3D and 4D). An analysis of LDH release into the growth medium showed a release of 30–40% of total LDH from the cells under conditions of As\(^{+3}\) exposure that resulted in a total loss of cell viability (Fig. 5D), a small but significant increase compared to the release of LDH by the parental cells (Fig. 5B).

**FIG. 2.** Effects of Cd\(^{+2}\) or As\(^{+3}\) on the viability of UROtsa parent and transformed cells. Confluent cultures of parent UROtsa cells and Cd\(^{+2}\)-transformed UROtsa cells (URO-CDSC) were exposed to various concentrations of Cd\(^{+2}\) for 24 (A), 36 (B), and 48 (C) h. Confluent cultures of parent UROtsa- and As\(^{+3}\)-transformed UROtsa (URO-ASSC) cells were exposed to various concentrations of As\(^{+3}\) for 24 (D), 36 (E), and 48 (F) h. Cell viability was determined by measuring the capacity of the cells to reduce MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] to formazan. Viability is expressed as percent of control. *Significant difference \((p < 0.05)\) compared to control.
Expression of MT Protein in UROtsa, URO-ASSC, and URO-CDSC Cells Exposed to Cd\(^{+2}\) and As\(^{+3}\)

The last goal of this study was to determine if metal induction of the MT protein was altered by the transformation of the UROtsa cells. To determine this, the level of MT protein was determined in UROtsa, URO-ASSC, and URO-CDSC cells over a 48-h time course of exposure to either 12 \(\mu\)M Cd\(^{+2}\) or 8 \(\mu\)M As\(^{+3}\). Lower doses of metals were used for this assay as at high doses, total protein estimations in cell lysates were not possible at 48 h due to extensive cell death. The results showed that the levels of the MT-1/2 protein increased for both the parental and URO-CDSC cells over the time course of exposure to Cd\(^{+2}\); however, there was no difference in the level of MT-1/2 protein accumulation between the parental and the transformed cells (Fig. 6A). For the As\(^{+3}\) exposed cells, the results showed that the level of MT-1/2 protein did not increase for either the parental or the ASSC cells over the time course of exposure and that there was no difference in MT-1/2 protein expression between the parental and As\(^{+3}\)-transformed cells (Fig. 6B). A corresponding analysis of the MT-3 protein showed only background levels of MT-3 protein expression in all three cell lines over the time course of exposure (data not shown).

DISCUSSION

The first goal of this study was to determine the mechanism of cell death when human urothelial cells were exposed to As\(^{+3}\) or Cd\(^{+2}\). The results demonstrated that the mechanism of cell death for UROtsa cells exposed to As\(^{+3}\) was primarily through apoptosis, but there was also a significant contribution due to necrotic cell death. That both mechanisms were operational in As\(^{+3}\)-induced cell death was determined using a combination of qualitative and quantitative measurements. The presence of apoptosis was confirmed by determining the existence of fragmented nuclei upon DAPI-staining of cells, the existence of a classic DNA ladder upon gel electrophoretic analysis, and the detection of cleaved caspase-3 and caspase-9 products in cell lysates by western analysis. These were all qualitative measurements and indicated only that an appreciable number of the cells were undergoing apoptotic cell death when exposed to As\(^{+3}\). A quantitative measure of the amount of necrotic cell death, and by extrapolation the amount of apoptotic cell death, was determined by the analysis of LDH release into the growth medium. This measurement is based on determining the amount of necrotic cell death by release of the cytoplasmic protein, LDH, into the growth medium. Specificity for necrosis is based on the fact that necrosis is mediated by the rupture of the cell membrane with release of cellular contents, while apoptosis proceeds by the formation of membrane-bound bodies which enclose the cytoplasmic contents which are removed by phagocytosis. Using LDH release, it was determined that approximately 30% of the cells died by necrosis, and from this it was assumed that the other 70% died by apoptosis, when parental UROtsa cells were exposed to As\(^{+3}\).

There is only limited information on the effect of inorganic arsenite on human urothelial cell death. Furthermore, extrapolation from animal models to humans is difficult since As\(^{+3}\) has been shown not to be carcinogenic in animal models, including the urinary bladder (Kitchen, 2001). However, DMA has been shown to serve as an effective substitute and can act as either a promoter or a complete carcinogen in the rat bladder (Life Sciences Research, 1989; Wanihuchi et al., 1996; Wei et al., 1999; Yamamoto et al., 1995). Examination of the urinary bladder using scanning electron microscopy of female F344 rats administered 100 ppm DMA showed leafy microridges, extensive pitting, increased separation of epithelial cells, exfoliation, and necrosis (Cohen et al., 2001; Shen et al.,
The technique of scanning electron microscopy was particularly effective in detecting the necrotic lesions on the bladder surface. This has led to the hypothesis that the necrotic insult is followed by increased proliferation from tissue repair, which leads to hyperplasia, and eventually the production of a bladder tumor. The technique of scanning electron microscopy would not be expected to identify apoptotic cells; however, the observation of appreciable cell exfoliation would be consistent with a concurrent apoptotic mechanism of cell death. Furthermore, there has been one report detailing that exposure to DMA does induce apoptosis in the rat urinary bladder (Jia et al., 2004). To the author’s knowledge, there has been no study that simultaneously detailed apoptosis and necrosis in the bladder urothelium of rats exposed to DMA. Thus, there is direct evidence that arsenic can elicit both necrotic and apoptotic cell death of urothelial cells in the rat bladder, and the findings using the UROtsa cells would be consistent with these observations.

That As\(^{3+}\) can produce both necrosis and apoptosis of urinary urothelial cells compliments recent studies which also show that As\(^{3+}\) itself stimulates the specific cell signaling transduction pathways involved in cell proliferation (Germolec et al., 1996). Similar to classic tumor promoters, arsenic has been shown to activate AP-1 and to induce the early response genes c-fos, c-myc, and c-jun. The UROtsa cells have also been shown to respond to As\(^{3+}\) by increasing their rate of cell proliferation and AP-1 activity (Luster and Simeonova, 2004;
As$^{+3}$ and Cd$^{+2}$-induced toxicity to be more resistant to As$^{+3}$. This is exactly what was found in the present study when the parental UROtsa cells were compared to the As$^{+3}$-transformed UROtsa cells, that is, the transformed cells were more resistant to As$^{+3}$, and the mechanism of cell death was shifted away from apoptosis toward a necrotic mechanism of cell death. Thus, under this expanded hypothesis, As$^{+3}$-induced cytotoxicity can set the stage for tissue repair in the urothelium and then subsequently act as a tumor promoter during the regeneration process through the stimulation of the regrowth of cells that have gained an increased resistance to As$^{+3}$. In the transformation studies with the UROtsa cells, the concentration of As$^{+3}$ eliciting initial cell toxicity was the same as that which allowed regrowth and transformation of the cells.

The mechanism underlying the increased resistance to As$^{+3}$ that develops during malignant transformation of the UROtsa cells is not known. The present study did examine MT expression since it is known that MT can associate with As$^{+3}$ and MT has been shown to be overexpressed in human archival specimens of bladder cancer (Yamasaki et al., 2006; Zhou et al., 2006a,b) and also in the tumor heterotransplants generated from the As$^{+3}$-transformed UROtsa cells (Zhou et al., 2006a,b). However, it was shown that the basal and induced expression of the MT protein was similar between the parental and As$^{+3}$-transformed cells, providing strong evidence that MT was not a mediator of the increased resistance to As$^{+3}$.

The results of the present study demonstrated that the responses of the UROtsa cells to Cd$^{+2}$ were very similar to those of the As$^{+3}$ exposed cells. These responses included the observation that Cd$^{+2}$ elicited both apoptotic and necrotic cell death of the parental UROtsa cells, that apoptosis was mediated by the caspase-3 pathway, and that the Cd$^{+2}$-transformed cells had enhanced resistant to Cd$^{+2}$ and were more likely to undergo necrotic cell death. It was also shown that MT was unlikely to participate in the increased resistance noted for the Cd$^{+2}$-transformed cells. There were also striking similarities between the two metals in the past study which detailed the malignant transformation of the UROtsa cells (Sens et al., 2004). In this study, the concentrations of the metal used for transformation were identical and the response of the cells to exposure were very similar, with two episodes of cell death followed by regrowth of the monolayer and subsequent malignant transformation of the cells. The tumor heterotransplants were also similar in histology with the only difference being that As$^{+3}$-transformed heterotransplants displayed more frequent areas of squamous differentiation. These similarities can be used to argue that Cd$^{+2}$ should be examined more aggressively as a possible risk factor in the development of bladder cancer, either alone or in combination with As$^{+3}$. As noted in the introduction, there is a substantial body of research that implicates arsenic in the development of human bladder cancer. In contrast, even though Cd$^{+2}$ has also been evaluated to be a serious human carcinogen it has not been as aggressively examined for its possible role in bladder cancer.

Simeonova et al., 2000). A dual role of As$^{+3}$ in being able to promote both cell death and cell proliferation in the normal urothelial cell would be consistent with this laboratory’s observations of the UROtsa cells during malignant transformation by As$^{+3}$ (Sens et al., 2004). In this study, it was shown that confluent cultures of UROtsa cells exposed to 1 μM As$^{+3}$ underwent cell death only after being continuously exposed to As$^{+3}$ for over 30 days. This was followed by regrowth of the few remaining viable cells to form a new monolayer in the continued presence of the identical level of As$^{+3}$. This was then followed by a second instance of overt cell toxicity where the remaining cells rapidly regrew to confluency and displayed an increased rate of cell growth compared to the parental cells. It was shown by the growth in soft agar and heterotransplantation into nude mice that these resulting cells had undergone malignant transformation. These observations would be consistent with a mechanism of initial As$^{+3}$-induced toxicity followed by the subsequent stimulation of the cells to proliferate once again to confluency. For this mechanism to be operational would require the cells that survive initial
The present results would suggest that such an examination might be warranted.

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