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Beclin-1 Expression in Normal Bladder and in Cd⁺² and As⁺³ Exposed and Transformed Human Urothelial Cells (UROtsa)

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Abstract

The expression of beclin-1 in normal human bladder and in Cd⁺² and As⁺³ exposed and transformed urothelial cells (UROtsa) was examined in this study. It was shown using a combination of real time PCR, western analysis and immunohistochemistry that beclin-1 was expressed in the urothelial cells of the normal bladder. It was also demonstrated that the parental UROtsa cell line expressed beclin-1 mRNA and protein at levels similar to that of the *in situ* urothelium. The level of beclin-1 expression underwent only modest alterations when the UROtsa cells were malignantly transformed by Cd⁺² or As⁺³ or when the parental cells were exposed acutely to Cd⁺² or As⁺³. While there were instances of significant alterations at individual time points and within cell line-to-cell line comparisons there was no evidence of a dose response relationship or correlations to the phenotypic properties of the cell lines. Similar results were obtained for the expression of the Atg-5, Atg-7, Atg-12 and LC3B autophagy-related proteins. The findings provide initial evidence for beclin-1 expression in normal bladder and that large alterations in the expression of beclin-1 and associated proteins do not occur when human urothelial cells are malignantly transformed with, or exposed to, either Cd⁺² or As⁺³.

Keywords

Arsenite; cadmium; bladder; beclin-1; and autophagy

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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INTRODUCTION

The UROtsa cells are an immortalized cell culture model of human urothelium that was developed through immortalization of a primary culture of human urothelial cells with the SV40 large T-antigen (Petzoldt et al., 1994, 1995). The UROtsa cells grow as a contact inhibited monolayer and are not tumorigenic as judged by the inability to form colonies in soft agar and tumors in nude mice. When adapted for growth in a serum-free medium, they show enhanced differentiation with a stratified morphology consistent with the structural features associated with the intermediate layers of the urothelium (Rossi et al., 2001). This laboratory has shown that exposure of the UROtsa cells to either Cd^{+2} or As^{+3} can directly cause malignant transformation of the cells (Sens et al., 2004). The tumor heterotransplants produced by the Cd^{+2} and As^{+3} transformed cells had histologic features consistent with human urothelial cell carcinoma of the bladder. The parental UROtsa cells and their Cd^{+2} and As^{+3} transformed counterparts have been used to define the mechanism of cell death following exposure of the cells to cytotoxic levels of Cd^{+2} and As^{+3} (Somji et al., 2006). In this study it was demonstrated that the parental UROtsa cells died by both apoptosis and necrosis when exposed to either metal. It was also shown that apoptosis was the more prominent mechanism of cell death in the parental cells, accounting for over 50% of cell death. For the transformed UROtsa cells, it was shown that they were more resistant to the toxic effects of both metals and that the apoptotic mechanism of cell death was decreased and necrosis increased compared to the parental UROtsa cells. Subsequently, a third mechanism of cell death, called autophagic cell death, has subsequently been proposed in the literature that can operate separately or in concert with necrosis or apoptosis (Kroemer and Levine, 2008; Maluri et al., 2009; Todde et al., 2009; Sinha and Levine, 2008). It has been suggested that arsenic salts can induce autophagic cell death in SV40 immortalized human urothelial cells (Chai et al., 2007).

Autophagic cell death has been characterized by the large scale sequestration of portions of the cellular cytoplasm in autophagosomes, which gives the cell a vacuolated appearance characteristic of autophagy (Kroemer and Levine, 2008). Transmission electron microscopy is used to identify autophagosomes as double-membrane vesicles that contain cytosol or morphologically identifiable cytoplasmic organelles. Autolysosomes arise from the fusion of autophagosomes and lysosomes which are characterized by a single membrane that contains degenerating organelles undergoing the process of digestion by lysosomal enzymes. The autophagy pathway is conserved among all eukaryotes, is active under homeostatic conditions to remove aged organelles, and many autophagy effectors (Atg proteins) as well as other protein regulators have been identified in the last decade (for review, Sinha and Levine, 2009). Among these effectors, beclin-1, has been shown to be an essential effector of autophagy (Sinha and Levine, 2009). Mice with a biallelic loss of beclin-1 are early embryonically lethal (Qu et al., 2003; Yue et al., 2003). Mice with monoallelic loss of beclin-1 have an increased incidence of spontaneous tumor formation (Qu et al., 2003; Yue et al., 2003), have abnormal proliferation of mammary epithelial cells (Qu et al., 2003), increased susceptibility to neurodegeneration (Pickford et al., 2008) and cardiomyopathy (Tannous et al., 2008). In humans, monoallelic deletions of beclin 1 are observed frequently in sporadic breast, ovarian and prostate cancer (Aita et al., 1999). Due to the essential role of beclin-1 in effecting the autophagic process in cells, the present study examines its expression in normal urothelium and in urothelial cells exposed to, and transformed by, Cd^{+2} and As^{+3} . The first goal was to determine if beclin-1 is expressed in normal bladder urothelium. The second goal was to determine if beclin-1 expression is altered by malignant transformation of urothelial cells by Cd^{+2} or As^{+3} . Lastly, to determine if beclin-1 expression was altered by acute exposure of urothelial cells to Cd^{+2} or As^{+3} .

MATERIALS AND METHODS

Human Bladder Specimen for Immunohistochemical and Molecular Analysis

Specimens of normal human bladder were obtained from five patients undergoing a cystectomy for bladder cancer. For molecular analysis, the samples were immediately snap-frozen in liquid nitrogen. For immunohistochemical analysis, paraffin blocks were routinely fixed in neutral buffered formalin for 16–18 hours. All tissues were transferred to 70% ethanol and dehydrated in 100% ethanol. Dehydrated tissues were cleared in xylene, infiltrated, and embedded in paraffin. All tissue acquisition was approved by the institutional review board (IRB) for human research.

Immunohistochemistry

Serial sections were cut at 3–5 μ m for use in immunohistochemical protocols. After deparaffinization, sections were immersed in preheated Target Retrieval Solution (Dako, Carpinteria, CA) and heated in a steamer for 20 min. The sections were allowed to cool for 30 minutes at room temperature and immersed into Tris Buffered Saline with Tween 20 (Dako) for 5–10 minutes. Beclin-1 was detected using the Dako Envision^{TM} + kit (Dako) with an anti-beclin rabbit polyclonal antibody (1:100) purchased from Cell signaling (Cell Signaling Technology, Beverly, MA). After antigen retrieval, the sections were treated with Peroxidase Blocking Reagent for 10 minutes at room temperature. The slides were incubated overnight with the primary antibody at 4°C after which they were rinsed with the wash buffer (Dako). The slides were then incubated with the Peroxidase Labelled Polymer for 30 minutes at room temperature, rinsed and developed using liquid diaminobenzidine for visualization of the reaction product. The slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene and coverslipped. Normal human breast and prostate tissue were used as positive control, and negative control consisted of omission of the primary antibody from the immunohistochemical sequence.

RNA and Protein Isolation from Human Bladder

Frozen tissue was ground to a powder under liquid nitrogen. Total cellular RNA was isolated from the frozen, powdered tissue using protocols supplied with TRI REAGENT^{TM} (MRC, Cincinnati, OH) as previously described by this laboratory (Garrett et al., 1998). Purity and concentration of each sample were determined by spectrophotometric assay. All RNA samples utilized demonstrated no evidence of degradation as determined by intact bands of 18S and 28S ribosomal RNA following electrophoresis on 1.2% agarose gels. Proteins were extracted from the powdered tissue by dissolving it in 2% SDS containing 50 mM Tris-HCl, pH 6.8, followed by boiling for 10 min. DNA was sheared by passing the tissue extract through a 23-gauge needle. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) before 100 mM dithiothreitol (DTT) was added to each sample.

Cell Culture

Stock cultures of the parental UROtsa cell line and UROtsa cell lines malignantly transformed by Cd⁺² and As⁺³ were maintained in 75 cm² tissue culture flasks using Dulbecco's modified Eagles' medium (DMEM) containing 5% v/v fetal calf serum in a 37°C, 5% CO₂: 95% air atmosphere (Rossi et al., 2001). Confluent flasks were sub-cultured at a 1:4 ratio using trypsin-EDTA (0.05%, 0.02%) and the cells were fed fresh growth medium every 3 days.

Visualization of DAPI-Stained Cells

Toxic effects of Cd⁺² and As⁺³ on the UROtsa cells was determined by visualization of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei as described previously by this laboratory (Somji et al., 2004). At the indicated time points, cell monolayers were washed twice with phosphate buffered saline (PBS), fixed for 10 min in 70% ethanol, rehydrated in 1ml PBS, and stained with 10μl DAPI (10μg/ml in distilled water). For analysis, each well was examined under epifluorescent illumination at 40x magnification on a Zeiss Axiovert 35 inverted microscope with SPOT RT Slider digital camera using Adobe Photoshop.

Real Time Analysis of Beclin-1 mRNA Expression

The expression of beclin-1 mRNA was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) using commercially available primers from Qiagen (Valencia, CA). Total RNA was purified from the cell lines as well as the tissue sample and 1 μg was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μL. Amplification of the cDNA was performed using the SYBR Green kit (Bio-Rad Laboratories) with 2μL cDNA and 0.2 μM primers in a total volume of 20 μL in an iCycler iQ real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence. Cycling parameters consisted of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, which gave optimal amplification efficiency. The expression levels for the autophagy genes in the normal and transformed cell lines and human bladder tissue were determined relative to the UROtsa parental cells using serial dilutions of this sample for the standard curve. The resulting relative levels were then normalized to the change in β-Actin expression evaluated by the same assay using the primers, sense: CGACAACGGCTCCGGCATGT, and antisense: TGCCGTGCTCGATGGGGTACT, with cycling parameters of annealing/extension at 62°C for 45s and denaturing at 95°C for 15s.

Western Analysis of Beclin-1, Atg-5, Atg-7, Atg-12 and LC3B Expression

Confluent cultures were harvested in 2% SDS and 50 mM Tris-HCl, pH 6.8, followed by boiling for 10 min and DNA shearing through a 23-gauge needle. Protein concentration was determined by the BCA protein assay before 100 mM DTT was added to each sample. Ten micrograms of total cellular protein was separated on a 12.5 % SDS-PAGE gel and transferred to a hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in Tris buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% (w/v) non-fat dry milk for 1 hour at room temperature. After blocking, the membranes were probed with the appropriate primary antibody diluted in blocking buffer overnight. The primary antibodies used were Beclin-1 (1:2000), Atg-5 (1:2000), Atg-7 (1:1000), Atg-12 (1:1000) and LC3B (1:1000). All primary antibodies were purchased from Cell Signaling Technology. After washing three times in TBS-T, membranes were incubated with the anti-mouse or anti-rabbit secondary antibody (1:10,000) in antibody dilution buffer for one hour. The blots were visualized using the Phototope-HRP Western blot detection system (Cell Signaling). To determine equal loading of samples, membranes were stripped of bound proteins by incubating in 100mM 2-Mercaptoethanol, 2% SDS, 62.5mM Tris HCl pH 6.7 at 50°C for 30 minutes. Membranes were washed twice with PBS in 0.5% Tween-20 at room temperature and blocked following the previously mentioned protocol. Membranes were reprobed using 0.2μg/ml of the anti β-Actin antibody (Abcam, Cambridge, MA) followed by incubation with a 1:10,000 dilution of the anti-mouse secondary antibody. The western blots were quantified using NIH Image J Software by determining the Integrated Optical Density (IOD) for each band and graphed relative to the UROtsa parent or control. Statistical analysis consisted of ANOVA with Tukey posthoc testing using GraphPad PRISM 4 software with a level of significance of $p < 0.001$.

RESULTS

Expression of Beclin-1 in Normal Urothelium

Five samples of human bladder obtained from a surgical procedure after completion of diagnostic needs were used to determine the expression and localization of beclin-1 protein (Fig 1A). The results demonstrated that the urothelium was moderately immunoreactive for beclin-1, with the suggestion of a gradient of increasing expression from the basal cell layer to the apical urothelial cells. The bladder stroma had limited expression of beclin-1, but there were scattered inflammatory and stromal cells throughout that were clearly immunoreactive for beclin-1. The illustration presented is from a specimen where normal urothelium was well-removed geographically from tumor tissue, but all five samples gave similar staining patterns for beclin-1 in areas of normal urothelium. Total RNA prepared from the sample used above to illustrate beclin-1 staining was shown to contain beclin-1 specific mRNA using real time PCR and beclin-1 specific primers (Fig 1B). A protein extract prepared from the same sample was shown to contain beclin-1 protein using western analysis and a beclin-1 specific antibody (Fig 1C). Lower molecular weight bands were noted on the western blot using beclin-1 and β -actin antibodies, respectively. These bands presumably are degradation products due to the length of time between surgical removal and preparation of the sample for research. The other four samples also showed beclin-1 mRNA and protein expression, but due to the extent of tumor involvement they might contain some contamination from urothelial cancer cells.

Basal Expression of Beclin-1 in Parental UROtsa Cells and UROtsa Cells Transformed by As^{+3} and Cd^{+2}

The laboratory has described the direct malignant transformation of the parental UROtsa cells by both As^{+3} and Cd^{+2} (Sens et al., 2004). Recent studies have isolated additional independent isolates of As^{+3} and Cd^{+2} transformed UROtsa cells (Somji et al., 2009; Cao et al., 2010). The parental UROtsa cells, the 6 independent isolates of the As^{+3} transformed cells, and the 7 independent isolates of the Cd^{+2} transformed cells were all assessed for their basal expression of beclin-1 mRNA and protein (Fig 2). The expression of beclin-1 mRNA was similar between the parental UROtsa cells and 4 of the 6 isolates of the As^{+3} , and 3 of the 7 isolates of Cd^{+2} , transformed UROtsa cells (Fig 2A). Two isolates of the As^{+3} transformed cells and 4 isolates of the Cd^{+2} transformed cells had elevated expression of beclin-1 mRNA (Fig 2A). The corresponding analysis of beclin-1 protein demonstrated that 4 of the 6 As^{+3} transformed isolates had a reduced expression of beclin-1 compared to parental UROtsa cells (Fig 2B). Beclin-1 expression in the other 2 isolates showed one to be identical to parental control and the other elevated compared to parent cells. Beclin-1 protein expression when compared to parent was elevated in 5 of the 7 isolates of Cd^{+2} transformed cells and reduced in two isolates (Fig 2B). A comparison of beclin-1 mRNA and protein expression among the isolates demonstrates that there is only a very weak correlation between the level of mRNA and the corresponding expression of beclin-1 protein. For the As^{+3} transformed UROtsa cells, only isolate #6 shows an increase in both beclin-1 mRNA and protein. For the Cd^{+2} transformed UROtsa cells, 4 of 7 isolates show an agreement between an elevated level of mRNA and increased protein expression, but the magnitude of the increase in protein does not follow the pattern of mRNA expression. The differences in the level of beclin-1 protein among all the isolates were modest, varying between a decrease of 50% and a 2-fold increase over control.

Beclin-1 Expression in Parental UROtsa Cells Exposed to Cd^{+2} and As^{+3}

The parental UROtsa cells were exposed to 1.0, 2.0 and 4.0 μM Cd^{+2} with extracts prepared at 8, 16, 24, 36 and 48 hrs of exposure for the analysis of beclin-1 protein. Similarly, to determine the effect of As^{+3} , the cells were exposed to 2.5, 4.5 and 6.0 μM As^{+3} with

extracts prepared at 4, 8, 16, 24, and 36 hrs of exposure for the analysis of beclin-1 protein. The concentrations and times of exposure were chosen such that no loss of cell viability would occur at the lowest 2 concentrations over the time course, while the highest concentration would cause at least a 50% loss of cell viability at the final point in the respective time course. The concentrations and times of exposure were based on data from previous studies from the laboratory (Somji et al., 2006) and cell viabilities were confirmed in the present study (data not shown). The results of this analysis demonstrated that there was only a minimal alteration in the level of beclin-1 protein when UROtsa cells were exposed to concentrations of Cd^{+2} and As^{+3} that elicited no loss of cell viability (Fig 3A, B). An interesting finding was that there did appear to be an effect on basal beclin-1 expression following addition of fresh growth medium to the cells. When the parental UROtsa cells were fed fresh growth medium to initiate the experiment, the beclin-1 protein levels in control cells increased for 24 hrs following the addition of fresh growth medium (Fig 4).

Beclin-1 Expression in Cultures of Cd^{+2} and As^{+3} Transformed UROtsa Cells Exposed to Cd^{+2} and As^{+3}

Two isolates of the Cd^{+2} and As^{+3} transformed UROtsa cells were assessed for beclin-1 protein expression when re-exposed to Cd^{+2} and As^{+3} . The concentrations and times of exposure were chosen from past studies which showed transformation altered subsequent exposure to Cd^{+2} and As^{+3} (Somji et al., 2006). The concentrations and times of exposure were chosen such that no loss of cell viability would occur at the lowest 2 concentrations over the time course while the highest concentration would cause at least a 50% loss of cell viability at the final point in the respective time course. The isolates for testing were chosen based on the phenotypic property of the cells being able to colonize the organs of the peritoneal cavity, with one isolate able to colonize and the other unable to colonize the peritoneal cavity (Somji et al., 2010; Cao et al., 2010). The results showed that although there were modest alterations in beclin-1 expression at selected time points, there were no significant alterations that corresponded to a dose response at any given concentration (Fig 5A–D).

Expression of Atg-5, Atg-7, Atg-12 and LC3B Proteins in Parental UROtsa Cells and UROtsa Cells Transformed by Cd^{+2} and As^{+3}

Employing the protein extracts described above, the expression of the Atg-5, Atg-7, Atg-12 and LC3B proteins were determined in the parental UROtsa cells and their Cd^{+2} and As^{+3} transformed counterparts. The Atg-5 protein expression was reduced between 10 and 85% in the Cd^{+2} transformed cells compared to control (Fig 6A). There was a marked reduction of the Atg-7 protein in all the Cd^{+2} transformed cell lines (Fig 6B). The Atg-12 protein was also reduced in 5 of the 7 Cd^{+2} transformed cell lines, with 2 being close to control levels (Fig 6C). The expression of the LC3B protein was similar to control in 4 of the 7 Cd^{+2} transformed cell lines, elevated in one isolate, and reduced in the other 2 isolates (Fig 6D). An examination of each protein among all the isolates failed to disclose any consistent relationship between the pattern of expression of any protein among the isolates. There was also no consistent pattern of expression of the proteins among the isolates when the expression was judged examining the entire group of proteins. For the As^{+3} transformed cell lines, the Atg-5 protein was increased in 5 of the 6 isolates and was at control levels in the remaining isolate (Fig 7A). The Atg-7 protein was elevated in 2 isolates, reduced in 3 isolates and unchanged from parental cells in the remaining isolate (Fig 7B). The Atg-12 protein was elevated in 2 isolates, reduced in an isolate and was unchanged in 3 isolates (Fig 7C). The LC3B protein was elevated in all but one of the As^{+3} transformed cell lines (Fig 7D). There was no consistent pattern of expression of the proteins within and among the isolates.

The extracts from the two isolates of Cd⁺² and As⁺³ transformed UROtsa cells that were re-exposed to Cd⁺² and As⁺³ and analyzed for beclin-1 expression above, were also assessed for the expression of the Atg-5, Atg-7, Atg-12 and LC3B proteins. The results showed that although there were modest alterations in the expression of each protein at selected time points, there were no significant alterations that corresponded to a dose response at any given concentration for any isolate (data not shown).

DISCUSSION

The initial goal of the present study was to determine if beclin-1 was expressed in the normal human bladder. A combination of real time PCR, western analysis and immunohistochemistry was used to show that beclin-1 is expressed in the urothelium of the bladder. The profile of immunoreactivity suggested that staining increased from the basal cells to the umbrella cells of the stratified urothelium. The staining with the beclin-1 antibody also showed that a few scattered stromal and inflammatory cells stained intensely for beclin-1 within the beclin-1 negative underlying muscular layer of the bladder. It is not possible to quantify the absolute level of beclin-1 expression in the bladder, but the level was similar to that present in the UROtsa parental cell line as judged by western analysis. The levels of beclin-1 are close between the tissue and cell line when one considers that the stromal layer of the tissue had minimal expression of beclin-1 as judged by immunostaining using the beclin-1 antibody. To the author's knowledge this is the first documentation of beclin-1 expression in human urothelium.

The two additional goals of the current study were motivated by the report which showed that beclin-1 expression was elevated in As⁺³ exposed human urothelial cells and that an increase in autophagy correlated with the increased expression of beclin-1 (Chai et al., 2007). This observation was extended in the present report using the immortalized, but not tumorigenic, UROtsa cell line to examine the relationship between beclin-1 expression and As⁺³ exposure. The UROtsa cell line was chosen for analysis since it has been directly malignantly transformed by exposure to both Cd⁺² and As⁺³ and the resultant cell lines shown to form tumor heterotransplants with preservation of a histology consistent with human urothelial cancer (Sens et al., 2004). In addition, multiple independently generated cell lines of both Cd⁺² and As⁺³ transformed cells have been generated and characterized for some basic phenotypic properties (Somji et al., 2010; Cao et al., 2010). An important feature of these additional cell lines was that all produced subcutaneous tumor heterotransplants, but only 2 of the 6 isolates of the As⁺³ transformed cell lines and 1 of the 7 isolates of Cd⁺² transformed cell lines were able to colonize organs of the peritoneal cavity following injection of tumor cells into the peritoneal cavity. Thus, the employment of this cell system allowed the examination of beclin-1 expression: in parental UROtsa cells; in UROtsa cells transformed by Cd⁺² or As⁺³; in Cd⁺² and As⁺³ transformed cells having different potentials to colonize peritoneal organ sites; and in UROtsa cells exposed to Cd⁺² or As⁺³. The results did show some alterations in the expression of the beclin-1 protein that reached significance; however, these were very modest alterations in the level of the beclin-1 protein between parental UROtsa cells and their malignantly transformed counterparts and between and among the transformed cell lines themselves. The small magnitude of these alterations would render it likely that they would lose significance if several identical experiments were performed at independent times of experiment initiation. The study was also extended to include the expression of the Atg-5, Atg-7, Atg-12 and LC3B proteins associated with later stages of the autophagic process. Similar to that found for beclin-1, there were only modest alterations in expression between parental UROtsa cells and their malignantly transformed counterparts, and between and among the transformed cell lines themselves. This finding provides initial evidence that large alterations in the expression of beclin-1 and associated

proteins do not occur when human urothelial cells are malignantly transformed with either Cd^{+2} or As^{+3} .

The acute exposure of the parental UROtsa cells to either Cd^{+2} or As^{+3} also resulted in only modest changes in beclin-1 protein expression and no alterations that could be viewed as a strong dose response relationship. This was also true for the other proteins associated with the autophagic process. However, these experiments did reveal what appeared to be an increase of beclin-1 protein expression in the parental UROtsa cells as a function of the cultures being fed fresh growth medium. This observation could be important since many cultured cells are on a 3 day feeding cycle and it is likely that they may be deprived of nutrients on the day prior to receiving fresh growth medium. The present finding suggests that cultured cells, especially when confluent, could be activating and de-activating the pathway of autophagy as a function of the feeding schedule of the cells. If true, this would complicate data interpretation in studies using cultured cells to explore the role of autophagy in cell death and survival. This might be especially true for malignant cells that have elevated growth rates and therefore high rates of nutrient utilization. The major reason for including Cd^{+2} in the proposed study was that it is a known carcinogen and its relationship with autophagy in the human bladder has not been examined previously. The potential role cadmium may play in bladder cancer is related to the increased incidence of bladder cancer in smokers and the high level of cadmium accumulation in humans that is known to occur through tobacco smoke (Satarug et al., 2010).

The present study does not rule out a role for beclin-1 in Cd^{+2} and As^{+3} exposed and transformed urothelial cells since it focuses solely on the level of expression. The interaction of beclin-1 with its binding partners could still be altered by exposure to Cd^{+2} and As^{+3} and this could have significant effects of cell survival and cell death.

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Figure 1.

Expression of beclin-1 in human bladder tissue and UROtsa cell lines. (A) Real time RT-PCR analysis of beclin-1 mRNA levels. mRNA levels were normalized to β -actin and are shown as relative mRNA levels $\pm$ SE for the UROtsa parent cell line, human bladder tissue, and UROtsa cell lines transformed with arsenic (URO As⁺³) and cadmium (URO Cd⁺²). (B) Western blot analysis of beclin-1 protein levels from the same cell lines and tissue shown in (A). Duplicate blots were stained for beclin-1 and β -actin as a control. (C) Immunohistochemical staining of beclin-1 in normal human urothelium. The urothelium was moderately immunoreactive for beclin-1, with the staining of superficial layers stronger than the basal layer. Some scattered inflammatory cells in the lamina propria were also positive for beclin-1(x 200).

Figure 2.

Baseline expression of beclin-1 in UROtsa cell lines. (A) Real time RT-PCR analysis of beclin-1 mRNA levels. mRNA levels were normalized to β -actin and are shown as relative mRNA levels $\pm$ SE for the UROtsa parent cell line and UROtsa cell lines transformed with arsenic (As) and cadmium (Cd). (B) Western blot analysis of beclin-1 protein levels relative to parent from the same cell lines shown in (A). Duplicate blots were stained for beclin-1 and β -actin as a control. The #'s identify the independent cell lines isolated by the exposure of UROtsa cells to As⁺³ and Cd⁺² as described by Cao and coworkers (2010) and Somji and coworkers (2010), respectively. Statistically significant compared to UROtsa parent (*), $p < 0.001$.

Figure 3.

Protein expression of beclin-1 in UROtsa Parent cell line treated with cadmium or arsenic. (A) Western blot analysis of beclin-1 protein expression relative to control at various time points with treatment of Cd⁺². Treatment with 1μM Cd⁺², 2μM Cd⁺², and 4μM Cd⁺² are represented by circle, triangle, and star symbols respectively. 36hr and 48hr treatments with 4μM Cd⁺² were omitted due to loss of cell viability. (B) Western blot analysis of beclin-1 protein expression relative to control at various time points with treatment of As⁺³. Treatment with 2.5μM As⁺³, 4.5μM As⁺³, and 6μM As⁺³ are represented by circle, triangle, and star symbols respectively. Duplicate blots were stained for beclin-1 and β-actin as a control. Protein levels are shown as the mean ±SE.

Figure 4. Growth effect on beclin-1 expression in UROtsa Parent cells. Integrated optical density of beclin-1 protein is shown at various time points.

Figure 5.

Protein expression of beclin-1 in UROtsa cells transformed with cadmium or arsenic. (A) Western blot analysis of beclin-1 protein expression relative to percent of control in UROtsa Cd #1 cells treated with Cd⁺². 36hr treatment with 12µM Cd⁺² and 16µM Cd⁺² were omitted due to loss of cell viability. (B) Western blot analysis of beclin-1 protein expression relative to percent of control in UROtsa Cd #7 cells treated with cadmium. 36hr treatment with 16µM Cd⁺² was omitted due to loss of cell viability. (C and D) Western blot analysis of beclin-1 protein expression relative to percent of control in UROtsa As #1 cells (C) and UROtsa As #6 cells (D) treated with As⁺³. 36hr treatment of As #6 (D) was omitted due to loss of cell viability. Duplicate blots were stained for beclin-1 and β-actin as a control. Protein levels are shown as the mean ±SE.

Figure 6.

Expression of autophagy proteins in UROtsa parent and UROtsa Cd²⁺ transformed cell lines. Western blot analysis of (A) Atg-5, (B) Atg-7, (C) Atg-12, and (D) LC3B protein expression relative to the UROtsa parent cells. Duplicate blots were stained for each autophagy protein and β-actin as a control. Protein levels are shown as the mean ±SE. Statistically significant compared to UROtsa parent (*), $p < 0.001$.

Figure 7.

Expression of autophagy proteins in UROtsa parent and UROtsa As⁺³ transformed cell lines. Western blot analysis of (A) Atg-5, (B) Atg-7, (C) Atg-12, and (D) LC3B protein expression relative to the UROtsa parent cells. Duplicate blots were stained for each autophagy protein and β -actin as a control. Protein levels are shown as the mean $\pm$ SE. Statistically significant compared to UROtsa parent (*), $p < 0.001$.