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Increased Neuron Specific Enolase Expression by Urothelial Cells Exposed to or Malignantly Transformed by Exposure to Cd⁺² or As⁺³

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Abstract

Neuron specific enolase (ENO2, γ -enolase) is a biomarker used to help identify neuroendocrine differentiation in tumors. This laboratory has shown that ENO2 might be a biomarker for exposure to cadmium and arsenite. In this study these observations are extended to the urothelial cell, where environmental exposures are strongly linked to urothelial cancer. The UROtsa urothelial cell line and its Cd⁺²- and As⁺³-transformed counterparts were used as the model. Acute exposure of the UROtsa cells to both As⁺³- and Cd⁺²-caused significant increases in ENO2 expression. Treatment with the histone deacetylase inhibitor was also shown to significantly increase the expression of ENO2 mRNA. The expression of ENO2 was significantly elevated in the Cd⁺²- and As⁺³-transformed UROtsa cells and tumor transplants. In contrast, ENO1, was unaffected by exposure to As⁺³ or Cd⁺². Immunofluorescence showed ENO2 associated with both the nucleus and cytoplasm and cytoplasmic ENO2 co-localized with ENO1. The findings extend the evidence suggesting a link between As⁺³ and Cd⁺² exposure and neuroendocrine differentiation in tumors. The results suggest that ENO2 might be a biomarker of human exposure to Cd⁺² and As⁺³ that operates through histone modification.

Keywords

arsenic; cadmium; urothelial cancer; UROtsa; enolase; biomarker

1. Introduction

This laboratory has previously shown that ENO2, also commonly referred to as neuron specific enolase, is induced by acute and chronic exposure of the breast epithelial cell line,

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MCF-10, to either As^{+3} or Cd^{+2} (Soh et al., 2011). ENO2 is one of a series of three immunohistochemical markers commonly used to define neuronal differentiation of tumors, the others being chromogranin A and synaptophysin (Ellis, 1995). Enolase is an enzyme of the glycolytic pathway that catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate (Wold, 1971). The enolase molecule exists as a dimer composed from three distinct subunits coded by separate genes which were originally designated as α (liver), β (muscle) and γ (brain) and now more frequently, as ENO1, ENO3 and ENO2, respectively (Schmechel et al., 1978; Kato et al., 1983; Haimoto et al., 1985). The $\alpha\alpha$ isoenzyme is present in all fetal tissues and most adult mammalian tissues. The $\beta\beta$ - and $\gamma\beta$ -enolase are found predominantly in skeletal and heart muscle. The $\gamma\gamma$ - and $\alpha\gamma$ -enolase are present mainly in nervous tissue and in tissues with neuroendocrine cells. When used as immunohistochemical markers, ENO1 antibody is against the α -isoform, ENO2 is against the γ -isoform, and ENO3 is against the β -isoform. The expression of ENO2 or neuron specific enolase has not been examined as extensively in bladder cancer as in other cancers such as breast and prostate. There are several immunohistochemical studies which show that ENO2 is not expressed in normal urothelium (Vinores et al., 1984; Haimoto et al., 1985; Mai et al., 1994; Bollito et al., 2005). For expression in urothelial cancer, one study shows no ENO2 immunostaining in urothelial cancer (Mai et al., 1994), while two others showed that 25% and 48% of urothelial bladder cancers expressed ENO2, respectively (Jacobsen et al., 1990; Bjerkeheggen et al., 1994). A recent proteomic study has shown ENO2 to be elevated in muscle-invasive urothelial cancer compared to specimens obtained from non-invasive cancers (Niu et al., 2011).

The finding that ENO2 is elevated by both acute and chronic exposure to As^{+3} or Cd^{+2} in the breast epithelial cell suggested that a corresponding examination in the urothelial cell would be warranted since the etiology of urothelial cancer is strongly linked to environmental exposures (Bischoff and Clark, 2009). Environmental exposure to As^{+3} has a strong association with urothelial cancer and increased incidence correlates to the same endemic areas of the world where populations were identified with arsenic-induced skin cancer (Chiou et al., 1995; Tsuda et al., 1995; Smith et al., 1998; Steinmaus et al., 2000; Luster and Simeonova, 2004; Cantor and Lubin, 2007). The high level of Cd^{+2} accumulation in individuals who smoke cigarettes, along with the strong association of bladder cancer and smoking, is the major factor indirectly implicating Cd^{+2} in the development of urothelial cancer (Satarug and Moore, 2004; Satarug et al., 2010). There are also several epidemiological studies that have implicated Cd^{+2} in the development of urothelial cancer (Siemietycki et al., 1994; Kellen et al., 2007). This laboratory employs the UROtsa cell line as a model to explore the relationship between As^{+3} and Cd^{+2} exposure and the development of urothelial cancer. The UROtsa cell line is an immortalized, but not tumorigenic model that retains features of transitional urothelium when propagated on a serum-free growth medium (Rossi et al., 2001). This cell line has been used to show that both Cd^{+2} and As^{+3} can cause the malignant transformation of human urothelial cells (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010). Tumors from these cell lines were shown to retain a morphology consistent with human urothelial cancer; the most prominent type of bladder cancer in western countries (Bischoff and Clark, 2009). In the present study, these cell lines and their transplanted tumors were used to determine the role of acute and chronic exposure to As^{+3} and Cd^{+2} on the expression of ENO1, ENO2 and ENO3.

1. Materials and Methods

1.1 Immunostaining for ENO1 and 2 in Normal Bladder and Transplanted Tumors

Tissue for the immunohistochemical analysis of ENO1 and 2 expression in human bladder were obtained from archival paraffin blocks that originated from previously completed patient diagnostic procedures. These archival specimens contained no patient identifiers and

use was approved by the University of North Dakota Internal Review Board. Tumor samples originating from the As⁺³ and Cd⁺² transformed UROtsa cell lines were pre-existing specimens from previous studies (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010). Tissues were routinely fixed in 10% 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. Tissue sections were cut at 3-5 µm for use in immunohistochemical protocols. Prior to immunostaining, sections were immersed in preheated citrate buffer pH 6.0 and heated in a steamer for 20 minutes. The sections were allowed to cool to room temperature and immersed into Tris buffered saline with Tween 20 (Dako, Carpinteria, CA) for 5 minutes. The primary antibody for the localization of ENO1 (Abcam Inc., Cambridge, MA, Cat # Ab54979) was used at a 1:400 dilution and for ENO2 (Abcam Inc., Cambridge, MA, Cat # Ab79757) was used at a 1:250 dilution. Liquid diaminobenzidine was used for visualization. Slides were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

2.1 Cell culture

Stock cultures of the parental UROtsa cell line were maintained in 75 cm² tissue culture flasks using Dulbecco's modified Eagle's 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 subcultured at a 1:4 ratio using trypsin-EDTA (0.05%, 0.02%) and the cells were fed fresh growth medium every 3 days. The isolation and growth of the 7 isolates of the Cd⁺² transformed UROtsa cells and 6 isolates of the As⁺³ transformed UROtsa cells have been described previously (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010). They were grown and maintained using identical conditions. Briefly, the arsenite and the cadmium transformed cells were grown and maintained in 75-cm² tissue culture flasks in DMEM containing 5% vol/vol fetal calf serum. The cells were routinely passaged at a 1:10 ratio upon reaching confluency as described previously (Sens et al., 2004). For acute exposure to arsenite and cadmium, the UROtsa parental cells were exposed to 1, 2 and 4 µM CdCl₂ or 2, 4 and 6 µM NaAsO₂ for 48 hr. The cadmium and arsenite solutions were prepared in water prior to dilution in cell culture medium.

1.2 Expression of ENO1, ENO2 and ENO3 mRNA and Protein in the UROtsa Derived Cell Lines and Transplanted Tumors

The total RNA and protein extracts used for the real time determination of ENO1, ENO2 and ENO3 mRNA expression and western analysis of ENO1 and ENO2 protein expression were pre-existing specimens from previous studies in this laboratory (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010; Larson et al., 2010). Briefly, total RNA was isolated using the TRI reagent protocol and protein samples were extracted in 2% SDS and 50 mM Tris-HCl, pH 6.8 as described previously (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010; Larson et al., 2010). The tumor heterotransplanted tissue that was snap frozen in liquid nitrogen was ground to a powder prior to RNA and protein extraction. The expression of ENO1, ENO2 and ENO3 mRNA was determined using ENO1, 2 and 3 specific primers obtained from Qiagen (Valencia, CA). The levels of ENO1, 2 and 3 mRNA was determined relative to the parental UROtsa cells using ENO-1, 2 and 3 standards to generate a standard curve. The expression levels of ENO-1, 2 and 3 were normalized to β-actin. The results are expressed in copy number/1000 β-actin transcripts or plotted as % change. The expression of ENO1 and 2 protein was determined by western blotting using 20 µg of total cellular protein on a 12.5% SDS-polyacrylamide gel using the above ENO1 and 2 antibodies at 1:100,000 and 1:2,000 dilutions, respectively. Anti-mouse IgG HRP-linked Ab was used to detect ENO-1 and anti-rabbit IgG, HRP-linked Ab used to detect ENO-2. The biotinylated molecular weight marker was detected with anti-biotin-HRP-linked antibody. The blots were visualized using the phototope-HRP western blot detection system.

1.3 Treatment of UROtsa Cells with 5-Azacytidine and Histone Deacetylase Inhibitor MS-275

The UROtsa cells were seeded at a ratio of 1:10 and the next day they were exposed to 0.5 and 1.5 μM 5-azacytidine (AZC) or the histone deacetylase inhibitor MS-275 at 1.0, 3.0, and 10 μM for three days until the cells reached confluency as described previously (Somji et al., 2011). Cells were then harvested for the determination of ENO2 mRNA expression.

1.4 Immunolocalization of ENO1 and ENO2 in the UROtsa Cell Lines

The cell lines were grown in 24 well plates containing 12 mm glass coverslips at 37° C, 5% CO₂. Cells at a nearly confluent density were then fixed and stained as described previously for ENO1 and ENO2 localization in MCF-10A cells (Soh et al., 2011). Briefly, cells were fixed in 3.7% buffered, methanol-free formaldehyde (Polysciences, Inc, Warrington, PA) for 15-20 mins at room temperature. Coverslips were then quenched of free aldehyde with 0.1 M NH₄Cl for 15 mins, followed by permeabilization with 0.1% Triton-X100 for 10 mins. Cells were stained for ENO1 (primary and secondary antibodies) followed by staining for ENO2. Staining for ENO1 or ENO2 was carried out by incubation for 45-60 minutes at 37° C with 0.5 $\mu\text{g}/\text{ml}$ of mouse anti-ENO1 or a 1:100 dilution of rabbit anti-ENO2 antibody. Primary antibodies were detected by incubating cells with 2.7 $\mu\text{g}/\text{ml}$ of Alexa-Fluor 594 goat anti-mouse IgG or 2.7 $\mu\text{g}/\text{ml}$ of Alexa-Fluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) for 45-60 minutes at 37° C. Controls consisted of coverslips treated with the appropriate secondary antibody(ies) only, staining for ENO1 or ENO2 alone with appropriate mouse or rabbit secondary antibodies as well as with in-appropriate rabbit or mouse secondary antibodies, respectively. All controls stained appropriately and negative controls had virtually no staining when photographed under the same settings that were used for experimental cells. For experiments determining the localization of ENO2 to the nucleus, ENO2 staining was carried out as indicated above followed by staining with a 5 μM solution of To-PRO-3 iodide (Invitrogen) for 30 mins at 37° C. All coverslips were mounted in ProLong Gold anti-fade reagent with DAPI (Invitrogen) for nuclear counter staining. Cells were observed and images captured using a Zeiss LSM 510 Meta Confocal Microscope with LSM 510 software (Carl Zeiss MicroImaging Inc). Images were obtained by capturing z-slices at a depth of 0.05 μm . DAPI images of the same fields were captured by epifluorescence.

1.5 Statistical Analysis

All cell culture experiments were performed in triplicate and the results are expressed as the standard error of the mean. Statistical analyses consisted of ANOVA with Tukey *post-hoc* testing performed by GraphPad PRISM 4.

2. Results

2.1 ENO1, 2 and 3 mRNA and Protein Expression in the Parental UROtsa Cell Line and the Cd⁺²- and As⁺³-Transformed Cell Lines

The expression of ENO1, ENO2, and ENO3 mRNA and ENO1 and 2 protein was determined for the parental UROtsa cell line and the 7 Cd⁺²- and 6 As⁺³-transformed cell lines. The parental UROtsa cell line was shown to express ENO1 mRNA at a level 5 to 10 fold higher than the β -actin housekeeping gene (Fig 1A). This level of expression was not significantly altered compared to the parental cell line for any of the 7 Cd⁺²-transformed cell lines and for only one of the 6 As⁺³-transformed cell lines, where expression was reduced over 50% (Fig 1A, 2A). Western analysis of ENO1 protein expression showed no significant differences between the parental UROtsa cells and their Cd⁺²- and As⁺³-transformed counterparts (Fig 1A, 2A). The level of ENO1 protein did not appear to be reduced in the

As⁺³-transformed cell line which appeared to have a reduction in ENO1 mRNA. The parental UROtsa cell line was shown to express ENO2 mRNA well below that of the β -actin housekeeping gene, being on the order of 1 transcript of ENO2 per 1,000 transcripts of β -actin (Fig 1B). The expression of ENO2 mRNA was increased in all the Cd⁺²-transformed cell lines (Fig 1B) and for 5 of the 6 As⁺³ transformed cell lines, with the increases being several fold when compared to the parental cell line (Fig 2B). Western analysis of ENO2 protein expression showed ENO2 to be elevated in all the Cd⁺²-transformed cell lines (Fig 1B) and in 3 of the 6 As⁺³-transformed cell lines (Fig 2B). The parental UROtsa cell line, as well as all the Cd⁺²- and As⁺³-transformed cell lines, were shown to express very low, near background, levels of ENO3 mRNA (Fig 1C, 2C). Due to these low levels of ENO3 mRNA expression, ENO3 protein was not determined for the cell lines.

Immunofluorescence was employed to determine the intracellular localization of ENO1 and ENO2 in the cell lines. All of the As⁺³ and Cd⁺² transformed cell lines showed a similar pattern of intracellular localization and one of the Cd⁺² transformed cell lines (Cd #6) is shown in Figure 3A, whereas the UROtsa parent is shown in Figure 3F. Within each cell line, all cells stained strongly for ENO1 (red) with the majority of ENO1 being localized to the cytoplasm. There was no difference in the intensity of ENO1 staining or localization between the parent UROtsa cells (Fig 3F) and the Cd⁺²- and As⁺³-transformed cell lines (Fig 3A). ENO1 was found to have both a diffuse cytoplasmic and diffuse nuclear localization in all the cell lines (Fig 3A, F). Within each cell line, all cells stained strongly for ENO1 with the majority of ENO1 being localized to the cytoplasm. There was no difference in the intensity of ENO1 staining or localization between the parent UROtsa cells and the Cd⁺²- and As⁺³-transformed cell lines. The intracellular localization of ENO2 (green) was strongly punctate with staining localized to the nucleus as well as to the cytoplasm. ENO2 had more cell-to-cell variation in staining intensity within the population of each respective cell line when compared to that found for ENO1, where staining was very uniform among the cells. The preferred localization of ENO2 in UROtsa cells appears to be within the nucleus. This is suggested by the observation that cells with lower levels of ENO2 staining have more nuclear localization compared to the cytoplasm; whereas, cells with higher levels of ENO2 staining have strong staining in both the nucleus and cytoplasm. The As⁺³- and Cd⁺²-transformed cell lines had stronger staining for ENO2 when compared to the parent UROtsa cell line, and therefore, had more ENO2 in the cytoplasm (Fig 3A, F).

Optimal z-series confocal slices of 0.5 μ m were captured for each transformed cell line (Fig 3B) and the parent (Fig 3G), and examined as single slices to determine if there was co-localization of ENO1 and ENO2 (Fig 3B, G). Co-localization of ENO1 and ENO2 was observed in many but not all of the cells. Co-localization was most often observed in the cytoplasm and only rarely in the nucleus. The degree of co-localization was primarily dependent on the levels of ENO2 in the cytoplasm. As a result, the As⁺³- and Cd⁺²-transformed cell lines were observed to have more co-localization of ENO1 and ENO2 in the cytoplasm than the parent UROtsa cells. To confirm that ENO2 did localize to the nucleus in UROtsa cells, cells were stained for ENO2 followed by staining the nucleus with To-PRO-3 iodide. Optimal z-series confocal slices of 0.5 μ m were captured and examined as single slices as well as orthogonal views of the z-series through the x- and y-planes. It was shown that the cells in both the As⁺³- and Cd⁺²-transformed populations (Fig 3C-E) as well as in the parent UROtsa cells (Fig 3H-J) contained ENO2 localized to the nucleus (Fig 3C-E, H-J). The orthogonal views (Fig 3D-E, I-J) show that ENO2 staining was contained within the nucleus structure itself. No nuclear localization differences, beyond differences in ENO2 staining levels, were observed between the transformed and parent UROtsa cell lines.

2.2 ENO2 mRNA and Protein Expression in the Parental UROtsa Cell Line Acutely Exposed to Cd⁺² and As⁺³

The parental UROtsa cell line was exposed to 1, 2 and 4 μM Cd⁺² or 2, 4 and 6 μM As⁺³ over a 48 hr time course to determine if acute exposure to these agents would increase ENO2 mRNA and protein expression. The concentrations and exposures were chosen from a previous study and the concentrations used caused no loss of cell viability except at the highest concentration and 48hr time point (Larson et al., 2010). This determination showed that exposure to all three concentrations of Cd⁺² caused a significant increase in ENO2 mRNA following 8 hrs of exposure and that these levels remained elevated at relatively constant levels over the next 36 hrs of the time course (Fig 4A). The reduction in ENO2 mRNA noted at the highest concentration of Cd⁺² at the 48 hr time point was accompanied by a loss of cell viability. The expression of ENO2 protein was also elevated compared to control cells in the majority of the samples with expression of ENO2 protein following behind that of ENO2 mRNA expression (Fig 4B). The level of ENO2 mRNA was also increased following acute exposure of the UROtsa cells to As⁺³, but compared to the Cd⁺² exposed cells the increase in mRNA expression occurred at later points in the time course and at elevated levels of exposure (Fig 4C). However, despite the lower level of ENO2 mRNA expression following As⁺³ exposure, these levels were translated into clear elevations of ENO2 protein expression (Fig D).

2.3 ENO2 mRNA Expression in Parental UROtsa Cells Following Treatment with Inhibitors of DNA Methylation and Acetylation

The UROtsa parental cell line was treated with the histone deacetylase inhibitor, MS-275, and the methylation inhibitor, 5-AZC, to determine the possible role of epigenetic modifications on ENO2 mRNA expression. This analysis demonstrated that treatment of the cells with MS-275 significantly increased the expression of ENO2 mRNA (Fig 5A). Treatment of the cells with 5-AZC has no effect on the expression of ENO2 mRNA (Fig 5B). For the time points and concentrations shown, there was no loss of cell viability compared to untreated cells (data not shown).

2.4 Expression of ENO1 and ENO2 in Tumor Transplants and Normal Human Bladder

The expression and localization of ENO1 and ENO2 was determined in tumor transplants generated from the Cd⁺²- and As⁺³-transformed cell lines. The expression of ENO1 mRNA varied among the tumors produced from the Cd⁺²-transformed cell lines, with some cell lines producing tumors with elevated levels of ENO1 mRNA expression and others similar to, or slightly below, that found in the corresponding cell lines (Fig 1A). There was no relationship between the level of ENO1 mRNA expression in the cell line and the level found in the tumor produced from that cell line. Western analysis showed relatively constant levels of ENO1 protein among all the tumors derived from the respective cell lines (Fig 1A). Immunostaining for ENO1 in the tumor cells was very strong in intensity and similar among all the tumors generated from the respective cell lines and a representative illustration is shown for the tumor generated by the Cd#1 cell line (Fig 6A). The staining intensity for ENO1 was uniformly higher in the tumor cells than the stromal cells associated with the tumors. Similar results were noted for the tumors generated from the As⁺³-transformed cell lines. The expression of ENO1 mRNA also varied among the tumors produced from the As⁺³-transformed cell lines, with some cell lines producing tumors with elevated levels of ENO1 mRNA, others similar to or below that found in the corresponding cell lines (Fig 2A). Likewise, there was no relationship between the level of ENO1 mRNA expression in the cell line and the level found in the tumor produced from that cell line. Western analysis showed relatively constant levels of ENO1 protein among all the tumors derived from the respective cell lines (Fig 2A). Immunostaining for ENO1 in the tumor cells was very strong in intensity and similar among all the tumor generated from the respective cell lines and a representative

illustration is shown for the tumor generated by the As#3 cell line (Fig 6B). The immunostaining profile of ENO1 was determined for three specimens of normal human bladder and all were shown to have urothelium with intense staining for ENO1 protein with lesser staining intensity for the stromal cells. A representative illustration is shown for one specimen (Fig 6C).

The expression of ENO2 mRNA was elevated in all the tumor transplants generated from the Cd⁺²-transformed cell lines (Fig 1B). Western analysis demonstrated relatively constant ENO2 protein levels in the tumor transplants (Fig 1B). All the tumors produced from the Cd⁺²-transformed cell lines stained with moderate intensity for the ENO2 protein and representative profiles are shown for tumors produced from 3 cell lines (Fig 6D, E, F). The staining for ENO2 is strong for the less differentiated peripheral tumor cells and infiltrating cords of tumor cells while areas with prominent squamous differentiation have negative or faintly positive staining for ENO2. Stroma associated with the tumor did not stain for ENO2. A similar pattern of ENO2 expression was found for the tumors generated from the As⁺³-transformed cell lines. The major exception being that the tumors derived from the As⁺³-transformed cell lines had an elevated expression of ENO2 mRNA (Fig 2B). Otherwise, the expression of the ENO2 protein by western analysis was similar (Fig 2B) as was the ENO2 immunostaining profile of the tumors (Fig 6G, H, I). The immunostaining profile of ENO2 was determined for three specimens of normal human bladder and all were shown to have negative to weak staining of ENO2 in the urothelial cells. A representative illustration is shown for one specimen (Fig 6J).

ENO3 mRNA was determined for the tumors derived from both the Cd⁺²- and As⁺³-transformed cell lines and was shown to have a very low expression (Fig 1C, 2C). The expression of ENO3 was not pursued to the level of protein expression.

3. Discussion

This laboratory has previously shown that acute and chronic exposure of the MCF-10A breast epithelial cell line to both Cd⁺² and As⁺³ increased the expression of ENO2 (Soh et al., 2011). This observation provided initial evidence that ENO2 might be a biomarker for environmental exposure to these two known pollutants and carcinogens. Furthermore, as detailed in the Introduction, the expression of ENO2 has been used extensively as one of three immunohistochemical markers to identify neuroendocrine differentiation in a wide range of tumors. The extensive use of ENO2 as a diagnostic marker in tumor diagnostics shows that its expression is altered during the development and progression of a variety of cancers. The overall goal of the present study was to extend the evidence that ENO2 might be a biomarker of environmental exposure to Cd⁺² and As⁺³ to human bladder cancer, where environmental exposure is known to be a major factor in disease causation (Bischoff and Clark, 2009). The model employed was the immortalized, but not tumorigenic, UROtsa cell line and its Cd⁺² and As⁺³ malignantly transformed counterparts (Rossi et al., 2001; Sens et al., 2004; Cao et al., 2010; Somji et al., 2010). In general, the findings in the present study reinforced and extended those found using the MCF-10A cell line. Identical to that shown previously for the MCF-10A cell line, acute exposure of the parental UROtsa cells to either Cd⁺² or As⁺³ resulted in an increase in the expression of the ENO2 protein. Evidence for a specific effect on ENO2 was also suggested by the finding that ENO1 protein levels were unaffected by acute exposure to Cd⁺² or As⁺³. The expression of ENO2 mRNA and protein was low, but detectable, in the parental UROtsa cell line under basal conditions. It was also shown that the level of ENO2 mRNA increased significantly when the parental UROtsa cells were treated with the histone deacetylase inhibitor MS-275, implication histone modification in ENO2 regulation. A similar finding was noted for the MCF-10A cell line. Metals are known to alter global gene expression patterns during metal induced

carcinogenesis with alterations in epigenetic regulations (Arita and Costa, 2009). Epigenetic alterations leading to over-expression or silencing of specific loci have been correlated to methylation/demethylation of CpG islands and post-translational modifications of histone tails within the promoters of altered genes. It is not known why a particular locus is silenced or over-expressed due to long-term exposure and/or transformation by metals. It is possible that long term Cd^{+2} - and As^{+3} exposure are causing epigenetic alterations resulting in the overexpression of ENO2. There are reports in the literature that ENO2 is not expressed in the urothelium of archival diagnostic specimens of human bladder (Vinores et al., 1984; Haimoto et al., 1985; Mai et al., 1994; Bollito et al., 2005). In the present study, the immunohistochemical determination of ENO2 expression in archival specimens of human bladder showed the urothelium to be near to, or slightly above, background levels of expression. The finding that the parental UROtsa cells had approximately 1 transcript of ENO2 mRNA per 1,000 transcripts of the β -actin housekeeping gene would be in agreement with a low *in situ* expression of ENO2 in normal urothelium. Thus, the results of the present study reinforce the concept that acute exposure to either As^{+3} or Cd^{+2} can increase the expression of ENO2 in epithelial cells.

An advantage in the present study was that the UROtsa cells exposed chronically to Cd^{+2} and As^{+3} , in addition to forming colonies in soft agar, were able to form tumors when transplanted subcutaneously in immune compromised mice (Sens et al., 2004; Cao et al., 2010; Somji et al., 2010). This allowed an analysis of ENO1 and ENO2 expression in the malignantly transformed cell lines and their tumor transplants. Similar to that found for the MCF-10A cells able to form colonies in soft agar, the Cd^{+2} - and As^{+3} -transformed UROtsa cells were shown to have increased expression of ENO2 protein compared to the untransformed UROtsa cell line. Likewise, specificity was shown for the ENO2 isoform over that of ENO1, since ENO1 was not increased as much in the transformed cell lines. An analysis of ENO1 and ENO2 expression in the transplanted tumors showed the level of ENO2 to be increased greatly over the level found in the corresponding cell line. Immunostaining of tumor tissue for ENO2 showed strong staining that was extensive and concentrated in the undifferentiated proliferating tumor cells. The uniform and extensive staining of ENO2 in the undifferentiated tumor cells proves evidence that cells expressing ENO2 may have an advantage in tumor initiation and subsequent growth. Indirect evidence for this is also provided by the finding the ENO2 localization by immunofluorescence in the tumor initiating cell lines was variable in expression with some cells having high expression of ENO2 and others a lower expression. This suggests that cells able to initiate the tumors when injected into immune compromised mice might be those expressing ENO2. An interesting aspect of the present study was that ENO expression could be assessed in 7 independent transformations of the UROtsa cell line exposed to Cd^{+2} and 6 independent transformations using As^{+3} . The finding that ENO2 was elevated in most of the cell lines, and all the transplanted tumors, provides evidence that the induction of ENO2 is a fundamental feature of Cd^{+2} - and As^{+3} -induced transformation and not a chance occurrence that arose during a single transformation event.

The present study also confirmed the observation in the Cd^{+2} - and As^{+3} -transformed MCF-10A cell lines that there was substantial nuclear localization of ENO2. This finding was extended in the present study to suggest that the nucleus might be the primary or preferred site for ENO2. This was suggested by the visual observation that when ENO2 expression was low in a given cell it tended to localize to the nucleus and when ENO2 expression was higher, it localized to both the nucleus and cytoplasm. The finding of nuclear localization also suggests that ENO2 may have a function other than participation in the cell's glycolytic pathway. ENO1 is the enolase family member that is associated with the glycolytic pathway in epithelial cells (Kato et al., 1983). The level of ENO2 expression found in the Cd^{+2} - and As^{+3} -transformed cell lines is far below that of ENO1 and would not

be expected to enhance the overall level of glycolysis. Very little is known regarding possible alternate functions of the ENO2 protein. It is known that ENO1 is a multifunctional protein and has functions beyond that of its central role in glycolysis (Pancholi, 2001). The most interesting of these regarding a nuclear localization is that ENO1 has been shown to have an alternative protein form known as the Myc binding protein, MBP-1 (Pancholi, 2001; Lung et al., 2010). The start codon for MBP-1 corresponds to 400 bp downstream of the ENO1 ATG and thus is considered to be an alternative translation initiation product of ENO1 mRNA. It does not appear that this alternate start site is present in ENO2 UTR sequence; however, there is a strong homology between the ENO1 and ENO2 protein sequences.

The finding that ENO2 has a nuclear localization in the Cd⁺²- and As⁺³-transformed cell lines might impact on the past and future use of this marker in tumor diagnosis. The rationale for this is that the nuclear localization of ENO2 was apparent only when the Cd⁺²- and As⁺³-transformed cell lines were examined by immunofluorescence and when ENO2 expression was relatively modest. Under these conditions the localization of ENO2 could be clearly distinguished between that of the cytoplasm and nucleus. However, in the tumor transplants derived from these cell lines, the cytoplasmic staining of ENO2 was of sufficient intensity to overwhelm and mask the ability to easily define the presence or absence of nuclear localization. This would also appear to be the case in the numerous reports that assess ENO2 expression as a neuroendocrine differentiation marker in human tumors where, when present, cytoplasmic staining of ENO2 is intense. Specifically for urothelial cancer, neither of the reports assessing ENO2 in archival diagnostic specimens addresses the possible significance of nuclear localization (Jacobsen et al., 1990; Bjerkehagen et al., 1994). This suggests that an examination might be warranted that focuses on the nuclear localization of ENO2 in archival diagnostic specimens of urothelial cancer. The findings confirm and extend the evidence suggesting a link between As⁺³ and Cd⁺² exposure and neuroendocrine differentiation in tumors. The findings suggest that ENO2 might be a biomarker of human exposure to Cd⁺² and As⁺³ that operates through histone modification.

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Highlights

- Transformation the normal bladder cell line, UROtsa, increased the expression of enolase-2.
- Cd^{+2} or As^{+3} induced the expression of enolase-2 in non-transformed UROtsa cells.
- An HDAC inhibitor induced the expression, indicating epigenetic control mechanisms.
- Enolase-2 could be potentially developed as a biomarker of metal-induced bladder cancer.

Figure 1. Expression of Enolase-1, -2 and -3 in Cd^{+2} -Transformed UROtsa Cells

Messenger RNA levels for the three isoforms of enolase were assessed using real-time PCR and gene specific primers on RNA isolated from cells in culture or from tumor tissue after tumor formation in nude mice. Expression levels are expressed as transcripts per 1000 transcripts of β -actin which was also assessed using real-time PCR. Expression of enolase protein isoforms was assessed with western blots using specific antibodies.

Figure 2. Expression of Enolase-1, -2 and -3 in As³-Transfected UROtsa Cells

Messenger RNA levels for the three isoforms of enolase were assessed using real-time PCR and gene specific primers on RNA isolated from cell in culture or from tumor tissue after tumor formation in nude mice. Expression levels are expressed as transcripts per 1000 transcripts of β -actin which was also assessed using real-time PCR. Expression of enolase protein isoforms was assessed with western blots using specific antibodies. *Indicates $p < 0.01$ from that of the unexposed cells.

Figure 3. Localization of enolase-1 and -2 in UROtsa transformed and parent cells and localization of enolase-2 to the nucleus

Cells were stained for enolase-1 (red) and for enolase -2 (green) in images A,B and F, G. Confocal images were taken through the z-series and then the z-plane images were stacked together and merged with the epifluorescent image of DAPI nuclear stain (blue) to form the images shown in A and F. A single z-series slice showing co-localization of enolase-1 and -2 (yellow) is shown in B and G. Localization of enolase-2 (green) with the nuclear stain To-Pro-3 iodide (red) is shown in C-E and H-J. Single z-series slices are shown in C and H while orthogonal views in the X- and Y-planes are shown in D, I and E, J respectively. The transformed UROtsa Cd#6 cell line is shown in A-E and the parent cell line is shown in F-J. The bars shown indicate the scale for images of both the transformed and parent cell lines; each bar = 10 μ m.

Figure 4. Expression of Enolase-2 in Normal Parental UROtsa cells Exposed to Cd²⁺ or As³⁺
 UROtsa cells were exposed to 1, 2, or 4 $\mu\text{M Cd}^{2+}$ or 2, 4, or 6 $\mu\text{M As}^{3+}$ for up to 48 hours of exposure. At the indicated time points, cells were harvested for RNA and protein. Expression levels of enolase-2 was assessed using real-time PCR for mRNA levels and western analysis for protein levels. Enolase-2 mRNA levels are expressed as fold change over that measured in unexposed UROtsa cells. *Indicates $p < 0.01$ from that of the unexposed cells.

Figure 5. Epigenetic alteration of Enolase-2 Expression

Normal UROtsa cells were exposed to 1, 3, or 10 μ M of the histone deacetylase inhibitor MS-275 for up to 72 hours, or to 0.1, 0.2, 0.5, 1, or 1.5 μ M 5-azacytidine for 24 hours. Messenger RNA levels were assessed using real-time PCR.

Figure 6. Immunohistochemistry of Enolase-1 and -2 Expression in Normal Human Bladder and Metal-transformed UROtsa cell heterotransplants in Nude Mice

Enolase-1 (A through C) and enolase-2 expression (D through J) was assessed by immunostaining with enolase-specific antibodies on formalin-fix, paraffin-embedded tissue samples. Shown for enolase-1 is A: Cd#1, B: As#3, and C: normal human bladder. Shown for enolase-2 is D: Cd#1, E: As#3, F: As#4, G: As#1, H: AS#4, I: As#5, and J: normal human bladder. For enolase-2 immunostaining, the cells with more prominent squamous differentiation in the center of tumor nests are negative or faintly positive for enolase-2 (*). The less differentiated peripheral cells or infiltrating cords are strongly positive for enolase-2 (+).