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Variation of Keratin 7 Expression and Other Phenotypic Characteristics of Independent Isolates of Cadmium Transformed Human Urothelial Cells (UROtsa)

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Abstract

This laboratory has shown that a human urothelial cell line (UROtsa) transformed by cadmium (Cd^{+2}) produced subcutaneous tumor heterotransplants that resemble human transitional cell carcinoma (TCC). In the present study, additional Cd^{+2} transformed cell lines were isolated to determine if independent exposures of the cell line to Cd^{+2} would result in malignantly transformed cell lines possessing similar phenotypic properties. Seven independent isolates were isolated and assessed for their doubling times, morphology, ability to heterotransplant subcutaneously and in the peritoneal cavity of nude mice and for the expression keratin 7. The 7 cell lines all displayed an epithelial morphology with no evidence of squamous differentiation. Doubling times were variable among the isolates, being significantly reduced or similar to the parental cells. All 7 isolates were able to form subcutaneous tumor heterotransplants with a TCC morphology and all heterotransplants displayed areas of squamous differentiation of the transitional cells. The degree of squamous differentiation varied among the isolates. In contrast to subcutaneous tumor formation, only 1 isolate of the Cd^{+2} transformed cells (UTCd#1) was able to effectively colonize multiple sites within the peritoneal cavity. An analysis of keratin 7 expression showed no correlation with squamous differentiation for the subcutaneous heterotransplants generated from the 7 cell lines. Keratin 7 was expressed in 6 of the 7 cell lines and their subcutaneous tumor heterotransplants. Keratin 7 was not expressed in the cell line that was able to form tumors within the peritoneal cavity. These results show that individual isolates of Cd^{+2} transformed cells have both similarities and differences in their phenotype.

INTRODUCTION

Cadmium is widely accepted as a human carcinogen and epidemiological studies have implicated the metal in the development of bladder cancer (1–3). Model systems of Cd^{+2} -induced bladder cancer would be valuable in elucidating the mechanism/s of Cd^{+2} carcinogenesis in this organ. A previous study from this laboratory has shown that both Cd^{+2} and arsenite (As^{+3}) can directly cause the malignant transformation of an immortalized, but non-tumorigenic, human urothelial (UROtsa) cell line (4). The determination of colony formation in soft agar was followed by demonstrating that the cells could form tumors when

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subcutaneously heterotransplanted into nude (immunocompromised) mice. The histology of the tumor heterotransplants produced by UROtsa cells malignantly transformed by both As^{+3} and Cd^{+2} had features consistent with those of a classical transitional cell carcinoma of the bladder. Transitional cell carcinoma is the fourth most common cancer in men and the fifth in women in western countries (5). An interesting feature of the tumors was that all the tumor heterotransplants contained prominent areas where the transitional cells had undergone squamous differentiation. Squamous differentiation is found infrequently within tumors from patients diagnosed with transitional cell carcinoma and its presence is associated with a poor prognosis (6–9).

The goal of the present study was to determine, using an identical protocol of cell transformation, if independent exposures of the UROtsa cell line to Cd^{+2} would result in the generation of malignantly transformed cell lines possessing similar phenotypic properties. In the present study, eight identical cell cultures of UROtsa cells were each exposed to $1 \mu\text{M}$ Cd^{+2} using the protocol previously described by this laboratory (4). Phenotypic characterization included morphology and doubling times of the transformed cell lines, the histology of subcutaneous heterotransplants in nude mice, and the ability to colonize internal organ sites following intraperitoneal injection of the cells into nude mice. Keratin 7 expression was chosen for characterization of gene expression since it has been shown in humans to be expressed in cells of the normal urothelium and that the expression may be altered as a result of malignant transformation (10–12). The expression of keratin 7 was also chosen for analysis due to the increased squamous differentiation noted in the tumors produced by the previous isolates of the Cd^{+2} and As^{+3} transformed UROtsa cells. A review of the literature suggests that few studies have examined the repeatability of the phenotypic and genotypic character of independent malignant transformations of a single cell line by one environmental agent.

EXPERIMENTAL PROCEDURES

Cell Culture

Stock cultures of the UROtsa cell line were maintained in 75 cm^2 tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM) containing 5% v/v fetal calf serum in a 37°C , 5% CO_2 : 95% air atmosphere (13). 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.

Cadmium-Induced Transformation of UROtsa Cells

The protocol used to malignantly transform the UROtsa cell line with Cd^{+2} has been detailed in an earlier report (4). An identical protocol was used in the present study. Eight parental cultures of UROtsa cells were grown to confluency in 25 cm^2 cell culture flasks and when confluent, each of the eight flasks were fed fresh growth media containing $1 \mu\text{M}$ Cd^{+2} (CdCl_2 , Sigma, St. Louis, MO). Following addition of Cd^{+2} , the cells were thereafter fed fresh growth media every three days that contained Cd^{+2} . The cultures were observed immediately before and 24 h after each feeding by light microscopy.

Growth in Soft Agar

Before testing for tumor growth in nude mice, all cultures were tested for their ability to form colonies in soft agar using a slight modification of the procedure described by San and coworkers (4,14). Briefly, 60 mm diameter dishes were prepared with a 5 ml underlay of 0.5% agar in DMEM containing 5% fetal calf serum. On top of the under layer was placed 2×10^4 cells in 1.5 ml of 0.25% agar in DMEM containing 5% fetal calf serum. The dishes were incubated at 37°C in a 5% CO_2 : 95% air atmosphere inside humidified plastic containers to prevent evaporation. Cultures were examined microscopically 24 h after plating to confirm an absence of large clumps of cells and thereafter at 7, 14 and 21 days after plating.

Subcutaneous Injection of Cd⁺² Transformed UROtsa Cell Lines to Determine Tumorigenicity in Nude Mice

To test for malignant transformation, the respective cultures that showed colony formation in soft agar along with the UROtsa parent cell line, were each inoculated subcutaneously (s.c.) at a dose of 1×10^6 cells in the dorsal thoracic midline of 5 nude (NCR-nu/nu) mice. Tumor formation and growth were assessed weekly. All mice were sacrificed by 10 weeks after injection or when clinical conditions dictated euthanasia. Tumor samples were paraffin-embedded, sectioned, stained with Hematoxylin and Eosin (H&E), and analyzed by light microscopy.

Intraperitoneal Injection of Cd⁺² Transformed UROtsa Cell Lines

To determine the ability of the transformed cell lines to colonize internal organs of the peritoneum, the Cd⁺² transformed cell lines and the parent were each injected intraperitoneally (IP) into 6 nude (NCR-nu/nu) mice. The IP injection was performed according to the online protocol of the American Association of Laboratory Animal Science learning library. A one inch 23 gauge needle was inserted in the abdominal cavity in the lower right quadrant and each mouse received 1×10^6 cells in 200 μ L of PBS. All the mice were euthanized at 53 days after injection when the tumors in one group became large and observable by visual examination of the abdomen. Necropsy was performed on each mouse according to the online dissection guide published by the National Institutes of Health (<http://www3.niaid.nih.gov/labs/aboutlabs/cmb/InfectiousDiseasePathogenesisSection/mouseNecropsy/>) with minor modifications. Briefly, at necropsy the general condition of the mouse was observed with determination of the weight, length (head to anus), and abdominal girth. The thoracic cavity, cranial cavity and especially the peritoneal cavity were examined carefully for gross tumor formation. At least 6 photographs were taken for each mouse including: an external dorsal view; external ventral view; ventral view with the skin opened; open abdominal cavity, open abdominal cavity with intestines removed; and, open abdominal cavity with the liver removed from the abdomen. All abdominal organs and associated tumor samples were formalin fixed and paraffin-embedded. Selected specimens were sectioned, stained with H&E, and analyzed by light microscopy.

Cell Growth

Growth curves of the malignantly transformed cells were obtained using the methylthiazolotetrazolium (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay following a 1:20 subculture of the cells (15).

Real Time Analysis of keratin 7 mRNA Expression

The expression of keratin 7 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 and tumor heterotransplants and 1 μ g was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μ L. Amplification of the cDNA was performed using the SYBR Green kit (Bio-Rad Laboratories) with 2 μ L cDNA and 0.2 μ M primers in a total volume of 20 μ L 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 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, which gave optimal amplification efficiency. The expression levels of keratin 7 in the transformed cell lines and tumor heterotransplants was determined relative to the UROtsa parental cells grown in serum-containing medium using serial dilutions of this sample for the standard curve. The resulting relative levels were then normalized to the change in beta-actin expression assessed by the same assay using the primers, sense: CGACAACGGCTCCGGCATGT and antisense:

TGCCGTGCTCGATGGGGTACT, with the cycling parameters of annealing/extension at 62°C for 45 s and denaturation at 95°C for 15 seconds.

Western Analysis of Keratin 7 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 bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) before 100 mM dithiothreitol (DTT) was added to each sample. Frozen tumor heterotransplant tissue was homogenized in the buffer as mentioned above and then was treated in the same way as described for the cell lines. Ten micrograms of total cellular protein was separated on a 12.5 % SDS-PAGE gel and transferred to a hybond-P polyvinylidene difluoride 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 h at room temperature. After blocking, the membranes were probed with a 1:1000 dilution of the Keratin 7 primary antibody (Abcam, Cambridge, MA) in blocking buffer over night. After washing three times in TBS-T, membranes were incubated with the anti-mouse secondary antibody (1:2000) in antibody dilution buffer for one hour. The blots were visualized using the Phototope-HRP Western blot detection system (Cell Signaling Technology, Beverly, MA.).

Immunostaining for Keratin 7

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. Serial 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. Keratin 7 was localized using a monoclonal antibody from Dako as the primary antibody at a 1:50 dilution. For tumor heterotransplants, the primary antibody was localized using the DakoCytomation ARK™ (Animal Research Kit), and Peroxidase. This system minimizes reactivity of secondary anti-mouse antibody with endogenous immunoglobulin that may be present in the heterotransplant-generated specimen. Liquid diaminobenzidine was used for visualization. Slides were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

Immunofluorescence Analysis

The UROtsa cells were grown in 24 well plates containing 12 mm glass coverslips at 37°C, 5% CO₂. Cells at a subconfluent density were then fixed and stained according to published protocols (16,17). Briefly, cells were fixed in ice-cold 100% methanol for 3–5 min at –20°C. Keratin 7 was detected via indirect immunofluorescence using a primary antibody to cytokeratin-7 from Abcam. The primary antibody was diluted to a concentration of 10.0 µg/ml and incubated on cells for 45–60 min at 37°C. Primary antibodies were detected using Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). The secondary antibody was diluted to a concentration of 4.0 µg/ml and incubated on cells for 45–60 min at 37°C. Duplicate coverslips stained for kertain-7 were prepared for all cell lines. Controls consisted of coverslips treated with secondary antibody only. Coverslips were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) for nuclear counter staining. Cells were observed and images were captured using a Zeiss LSM 510 Meta Confocal Microscope with LSM 510 software (Carl Zeiss MicroImaging Inc). Images were composed by capturing z-slices at a depth of 0.5 µm, stacking the z-slices together, and merging with the DAPI image of the same field so all cells in the field could be identified. Image processing and compilation was performed using Adobe Photoshop CS2.

RESULTS

Cadmium-Induced Transformation of Independent Cultures of UROtsa Cells

The results of the current protocol followed an experimental course very similar to that described previously for the malignant transformation of single cultures of UROtsa cells by Cd^{+2} (4). The 8 independently initiated cultures of UROtsa cells exposed to $1\mu\text{M}$ Cd^{+2} each underwent an initial cycle of cell death followed by re-growth of the monolayer. The cells from the resulting monolayers were not able to form colonies in soft agar. The re-growth of the monolayer was followed by an additional cycle of cell death and in this instance, 6 of 8 cultures were able to proliferate and form a confluent monolayer of cells. The other 2 cultures did not recover despite continued feeding for several months. The 6 surviving UROtsa cell cultures were able to form colonies in soft agar (Table 1). These resulting cultures were able to be subcultured at 1:20 ratios and multiple flasks of cells were prepared and processed for long-term storage under liquid nitrogen. Combined with the isolate from this laboratory's previous study, 7 independent cultures of Cd^{+2} transformed UROtsa cells were available for further characterization. The isolate from the previous study was used at an identical passage number following initial isolation as the new isolates generated in the current study.

Cell Doubling Times, Light Level Microscopy, Subcutaneous Heterotransplantation and Tumor Histology

The determination of doubling times for the Cd^{+2} transformed cell lines demonstrated 5 of the 7 lines to have doubling times significantly shorter than the UROtsa parental cell line (Table 1). The other 2 Cd^{+2} transformed cell lines had doubling similar to that of the parental cells. The light level morphology of the Cd^{+2} transformed cell lines showed that all the lines possessed an epithelial morphology and that this morphology was similar among the lines (Figure 1). The cultures could be subcultured at a 1:20 ratio and formed cell monolayers with little evidence of a loss of contact inhibition of growth or the formation of multilayered foci of cells. There was no obvious correlation of morphology at the light level of microscopy with the doubling times of the cell lines.

Each transformed cell line and the parent UROtsa cell line was inoculated s.c. at a dose of 1×10^6 cells in the dorsal thoracic midline of 5 nude mice to confirm that the cultures that were able to form colonies in soft agar were also capable of forming tumors. The mice were sacrificed 10 weeks after inoculation except for one mouse in the UTCd#1 group that was sacrificed in week 8 due to displaying lethargic behavior and a large tumor masse. There was tumor formation in each group of mice for each of the individual isolates of the Cd^{+2} transformed cell lines (Table 1). The parent line did not form a tumor in any of the 5 mice. There was some variation in the success of heterotransplantation among the cell lines; with tumor heterotransplants noted in 5 of 5 mice for one cell line, 4 of 5 mice for two cell lines, 3 of 5 mice for one cell line, and 2 of 5 mice for three cell lines. There was no correlation of the ability of the cell lines to form heterotransplants with cell doubling times or cell morphology of the tumor heterotransplants. These results demonstrated that all the Cd^{+2} transformed cell lines were capable of forming subcutaneous tumors in nude mice.

The subcutaneous tumor heterotransplants generated from the 6 new isolates of Cd^{+2} transformed cells had a very similar histology to that described previously for UTCd#1 (4). In general, the tumors were composed of infiltrating masses or nests of moderately differentiated cells with stratification of the malignant phenotype from the exterior to the central portion of the neoplastic masses (Figure 2). The peripherally located cells displayed less differentiation, with hyperchromatic nuclei, a higher nuclear to cytoplasmic ratio, and frequent mitotic profiles. All the isolates displayed a tumor histology that was similar to that expected of an invasive transitional cell carcinoma. Also in agreement with the previous study was the finding that all

the isolates contained areas of squamous differentiation of the transitional cells that was localized to the central or superficial cells (Figure 2). In contrast to the previous study, it was found that the degree of squamous differentiation, while constant within the tumors produced by each isolate, varied in prominence among the individual isolates (Table 1). The degree or prominence of squamous differentiation could be grouped into 3 patterns (Figure 2). A prominent pattern of squamous differentiation is defined as a histology profile that displayed frequent keratin pearls, the presence of numerous intercellular bridges, and stratification with readily identifiable keratohyaline. Cytoplasmic vacuoles of the middle and superficial squamous layers were absent or very rarely seen in a prominent pattern of squamous differentiation (Figure 2 A–F). In contrast, a mild pattern of squamous differentiation was characterized by a histology that displayed no or very rare keratin pearls, intercellular bridges and stratifications with keratohyaline (Figure 2 J–L). In tumors with mild squamous differentiation, there were prominent cytoplasmic vacuoles or clearance in the cells of middle or superficial layer. An intermediate pattern of squamous differentiation was assigned to tumors between these two patterns. Tumors with an intermediate squamous differentiation pattern displayed infrequent, but present, profiles of keratin pearls, intercellular bridges, and stratifications with keratohyaline. There were rare profiles of cytoplasmic vacuoles in the cells of the middle or superficial layer (Figure 2 G–I). The pattern of the squamous component did not correlate with doubling times or the success rate of tumor heterotransplantation.

Ability of the Cd⁺² Transformed Cell Lines to Colonize Intraperitoneal Organs

Each cell line, including the parent, was inoculated at a dose of 1×10^6 cells into the peritoneal cavity of 6 nude mice to determine the ability of each cell line to colonize (seed) the organs located within the peritoneal cavity. The mice in all experimental groups were euthanized 53 days after injection, a time when visible inspection of the abdomen revealed one of the experimental groups to have substantial tumor growth. For the Cd⁺² transformed cell lines, only the UTCd#1 cell line was able to colonize sites within the peritoneal cavity, with the other 6 cell lines, including the parent, showing no tumor formation in any mouse within any group. Four of six mice inoculated IP with the UTCd#1 cell line formed tumors within the peritoneal cavity and those with tumor formation all showed extensive tumor nodules, numbering in the hundreds, within the peritoneal cavity (Table 1). The organs and tissues within the peritoneal cavity were also removed from each mouse and again examined for the gross presence of tumor nodules. The peritoneum was first examined for an injection site tumor that can form between the skin and external surface of the peritoneum and is by definition a subcutaneous tumor. These occur due to leakage of a small amount of cells during the IP injection and serve as a valuable control that viable cells were injected IP into the abdomen. At least 67% of the mice from all experimental groups except the parent had a small, but readily identifiable subcutaneous injection site tumor. These subcutaneous tumors were not counted as a tumor of the peritoneum when analyzing if tumor formation occurred within the peritoneal cavity following IP injection.

Only the UTCd#1 cell line formed tumors within the peritoneal cavity (Table 1). Four of the mice from this group of six had numerous nodules on gross examination of the abdominal cavity. The organ sites within the abdominal cavity that were visually examined for the presence of tumor nodules included the omentum, pelvic cavity, liver, stomach, spleen, diaphragm, and kidney. Again, no evidence of tumor formation was noted for mice in any of the 7 experimental groups, except the 4 mice from the UTCd#1 group that showed gross evidence of IP tumor formation. In all of these 4 mice, tumors were found distributed widely in the peritoneal cavity at the following locations: on the internal surface of the peritoneum and the greater omentum along the greater curvature of the stomach; the superficial pelvic cavity just inferior to the intestinal loops; the deep pelvic cavity (which requires removal of the intestinal loops) with nodules located in the pelvic part of the retroperitoneum with

attachment to the posterior abdominal wall; the liver located at the hepatic hilum between the liver and the lesser curvature of the stomach; the spleen and pancreas with tumor nodules located between the greater curvature of the stomach and spleen and those that grew around the spleen; the diaphragm with tumors identified as thickened with a whitish appearance or when a tumor nodule can be found on the abdominal surface of the diaphragm; the intestine with tumor nodules located in the mesentery or serosal surface of intestinal tract; and the kidney with tumor nodules growing around the kidney in the retroperitoneum. These results demonstrate that the individual Cd⁺² transformed cell lines varied in the ability to colonize sites within the peritoneal cavity.

Keratin 7 Expression in Subcutaneous and Intraperitoneal Tumor Heterotransplants Generated from the Cd⁺² Transformed Cell Lines

Immunohistochemistry was used to determine the expression and localization of keratin 7 in tissue sections from the formalin-fixed, paraffin embedded tissues of the s.c. and IP tumor heterotransplants (Figure 3 A–G). The results demonstrated that 6 of the 7 Cd⁺² transformed cell lines generated subcutaneous tumor heterotransplants that had substantial immunostaining for keratin 7 (Figure 3 B–G). The sole exception to this was the UTCd#1 cell line that produced subcutaneous tumor heterotransplants that demonstrated little, if any, expression of keratin 7 (Figure 3 A). For the heterotransplants that exhibited immunostaining for keratin 7, expression was uniform among the heterotransplants with approximately 40% of the cells in each tumor highly stained for keratin 7. In all instances, keratin 7 expression was localized to the cytoplasm. For all the heterotransplants, there was no direct correlation between keratin 7 expression and the squamous differentiation of the tumor. In general, keratin 7 expression was localized to areas of the tumor that showed minimal evidence of squamous differentiation. The IP tumor nodules from the UTCd#1 cell line that produced subcutaneous heterotransplants that did not express keratin 7 were also assessed by immunostaining for the presence of keratin 7. IP tumor nodules localized on the internal surface of the peritoneum and those associated with the internal organs were all noted to have minimal, if any, immunoreactivity for keratin 7 (Figure 3 I, J for examples). These IP specimens were also noted to have only rare profiles of squamous differentiation.

The expression of keratin 7 mRNA and protein was also determined on extracts prepared from the subcutaneous tumor heterotransplants generated from the 7 Cd⁺² transformed cell lines (Figure 4 A, B). Six of the 7 tumor heterotransplants expressed high levels of keratin 7 mRNA. In contrast, expression of keratin 7 mRNA was at a background level of detection in extracts prepared from tissues of the tumor heterotransplants generated by the UTCd#1 cell line. These results are in agreement with the immunostaining profiles for keratin 7 described above. Also in agreement with keratin 7 immunostaining was the demonstration by western analysis that all the heterotransplants expressed high levels of keratin 7 protein, except the heterotransplant generated from the UTCd#1 cell line, which had markedly reduced expression of keratin 7. The levels of keratin 7 mRNA and protein were not determined for tumor nodules produced by IP injection of the UTCd#1 cell line as all the tissue was processed for microscopic analysis.

Keratin 7 Expression in the Cd⁺² Transformed Cell Lines

The expression and localization of keratin 7 was determined on the parental UROtsa cells and the 7 Cd⁺² transformed cell lines. The expression of keratin 7 mRNA and protein, as determined by real time PCR and western analysis, in extracts from the 7 Cd⁺² transformed cell lines was similar to that found for the tumor heterotransplants (Figure 4 C, D). Keratin 7 mRNA and protein was elevated in the 6 Cd⁺² transformed cell lines that produced subcutaneous tumor heterotransplants that were shown to be immunoreactive for keratin 7 and to express keratin 7 mRNA and protein by real time PCR and western analysis. In contrast, there was no expression of keratin 7 mRNA or protein in extracts prepared from the UTCd#1 cell line, a cell line that

produced subcutaneous tumor heterotransplants that had minimal, if any, expression of keratin 7 mRNA or protein. The parental UROtsa cells were demonstrated to express both keratin 7 mRNA and protein.

Keratin 7 was localized within the parental UROtsa cells and cells of the Cd^{+2} transformed cell lines using immunofluorescent microscopy. The results showed that only a small percentage (10 to 20%) of the parental UROtsa cells had keratin 7 organized into intermediate filaments. However, some of the parental UROtsa cells that did contain keratin-7 positive intermediate filaments displayed strongly stained, thick filaments (Figure 5 A). Six of the 7 cell lines malignantly transformed with Cd^{+2} had strong expression of keratin 7 filaments in over 90% of the cells (Figure 5 C–H). The exception was the UTCd#1 Cd^{+2} transformed cell line that displayed an absence of keratin 7 filaments in all the cells examined and only an occasional cell with diffuse staining of keratin 7 in the cytoplasm (Figure 5 B). Higher magnifications of these observations are shown for the UTCd#1, UTCd#3 and UTCd#6 cell lines (Figure 6).

DISCUSSION

Bladder cancer is a significant health issue both globally and within the United States. In the United States in 2007, there were approximately 67,160 new cases of bladder cancer and 13,760 deaths attributable to this disease (18). The prevalence in the USA is approximately 490,000 cases whereas the worldwide estimate of bladder cancer is well over one million cases. Urothelial (transitional cell) carcinoma (TCC) is the predominant type of bladder tumor (approaching 100%) in the United States. Roughly, 70% of newly diagnosed cases are superficial disease with no invasion of muscle (18). The major indicator of a poor prognosis is the invasion of the underlying muscle of the bladder. The development of bladder cancer is strongly linked to environmental factors. In early studies of the disease, most cases were found associated with workplace exposure to bladder carcinogens, such as aromatic amines and polycyclic hydrocarbons that are encountered in dye and wood product industries (19). More recent studies have defined an association between cigarette smoking and bladder cancer, with some reports suggesting a two- to four-fold increased risk and that 50% of the bladder cancers in men would not occur in the absence of cigarette smoking (20,21). The number of cigarettes smoked, degree of inhalation, type of tobacco, use of filters, and smoking cessation have all been shown to have specific relationships with the development of bladder cancer (22). In a recent estimate, workplace exposure accounted for 5–15% of European male cases while cigarette smoking was identified to be a predominant risk factor (23). In a Spanish case-control study of 1,219 newly diagnosed cases and 1,271 controls, cigarette smoking accounted for nearly all excess risk in men (24). The strong association of smoking with bladder cancer supports a role for cadmium in the development of bladder cancer. It is a well-established fact that smokers have markedly elevated levels of cadmium compared to non-smokers due to the bio-accumulation of cadmium in the tobacco leaf and its subsequent absorption from tobacco smoke; providing an indirect link between cadmium and the development of bladder cancer (25–28).

There is also epidemiological evidence for a role of cadmium in the development of bladder cancer. In a recent study, 172 patients with bladder cancer with a mean blood cadmium level of 1.1 $\mu\text{g/L}$ were compared with 395 controls that had a mean blood cadmium of 0.7 $\mu\text{g/L}$ (1). It was shown that there was a 5.7 fold increase in bladder cancer risk as blood cadmium rose from the lowest tertile to the highest. Furthermore, the bladder cancer risk estimate was corrected for gender, age, smoking habits and workplace exposures. Additional indirect evidence comes from the fact that cadmium is known to have an extremely long half life; covering many decades once absorbed into the human body. In the above study by Samanic and co-workers (24), it was also shown that the risk of bladder cancer fell by 30% in the first

4 years after smoking cessation; while an additional 20 years needed to elapse for a further 30% reduction in risk. The finding of a decade-long carrying over effect provides evidence that cancer-causing agents undergoing rapid clearance from the body are less likely to play a key role in the development of bladder cancer. Rather, known carcinogens, such as cadmium, that have a decade long extended half-life are more likely to be retained in bladder tissues over an extended period and participate in the development of bladder cancer. Indeed, in a 20-yr Belgian cohort, Nawrot and co-workers (29) noted that blood and urinary cadmium levels fell slowly with a yearly decline rate of 1.8% and 3.4%, respectively.

In an effort to produce a model for environmentally induced bladder cancer, this laboratory has shown previously that Cd^{+2} exposure can cause the direct malignant transformation of the UROtsa human urothelial cell line and that the histology of the tumor heterotransplants displayed features consistent with those of a transitional cell carcinoma of the bladder (4). The initial goal of the present study was to determine if multiple independent isolates of the UROtsa cell line malignantly transformed by Cd^{+2} would have similar phenotypic properties. The most readily identifiable phenotypic difference among the 7 cell lines transformed by Cd^{+2} was the wide variation in cell doubling times; which varied from those that were unchanged to those that were markedly decreased compared to those of the parental cell line. In contrast, the results showed that all 7 independent isolates of the Cd^{+2} transformed UROtsa cells formed subcutaneous tumor heterotransplants with features very similar to those reported previously for the initial isolate (4). The only distinguishing characteristic among the independent isolates were modest differences in the relative degree of squamous differentiation of the transitional cells comprising the subcutaneous tumors. The degree of squamous differentiation within the TCC background varied from mild to prominent among the independent isolates and did not correlate with differences in cell doubling times. In addition, all the cell lines displayed a light level morphology consistent with a monolayer of epithelial cells with no obvious squamous differentiation such as stratification and terminally differentiated cells. The cultures were grown on standard growth medium and not on keratinocyte growth media that contains reduced levels of calcium.

In contrast to subcutaneous injection of the cell lines, there was a marked difference in the ability of the 7 independent Cd^{+2} transformed cell lines to form tumor heterotransplants when injected into the peritoneal cavity of the nude mice. Only 1 of the 7 Cd^{+2} transformed cell lines could colonize the internal organs of the mouse, producing hundreds of tumor nodules within the peritoneal cavity. The other 6 Cd^{+2} transformed cell lines formed no visible tumor nodules within the peritoneum nor were microscopic nodules found on examination of H&E stained tissue sections of the organs within the peritoneal cavity and the inner surface of the peritoneal membrane (data not shown). The 6 cell lines that did not form tumors within the peritoneal cavity did form subcutaneous tumors at the injection site between the skin and peritoneal membrane. The parental UROtsa cells were unable to form colonies in soft agar or tumors when injected subcutaneously or into the peritoneal cavity of nude mice. The finding that one Cd^{+2} transformed cell line was able to form tumors within the peritoneal cavity is important, since the ability to colonize peritoneal organs is of significance in bladder cancer since these cancers tend to spread locally. Approximately 80% of high grade transitional cell cancers are invasive. Aggressive tumors may extend only into the bladder wall; however, the more advanced stages invade the adjacent prostate and seminal vesicles in males, and the ureters and retroperitoneum in both males and females, and some produce fistulous communications to the vagina or rectum. Approximately 40% of these deeply invasive tumors metastasize to regional lymph nodes. Hematogenous dissemination, principally to the liver, lungs and bone marrow, generally occur late in bladder cancer and only with highly anaplastic tumors. Thus, the phenotypic differences among the Cd^{+2} transformed cell lines and their tumor heterotransplants should provide an excellent model platform for further genomic and proteomic analysis of gene expression.

The last goal of the study was to begin to characterize the expression of the keratin gene family due to the finding of squamous differentiation within the tumor heterotransplants derived from the Cd⁺² transformed cell lines. Keratin 7 was chosen for analysis since it is expressed in all epithelial cells of the normal urothelium (12) and its expression has been reported to be altered in bladder cancer (10,11). The initial step in analysis was the histochemical staining for keratin 7 in the tumor heterotransplants generated from each of the 7 Cd⁺² transformed cell lines. It was demonstrated that keratin 7 staining was expressed focally in 6 of the 7 cell lines, with heterotransplants from the UTCd#1 cell line being negative for keratin 7 expression. Since all tumor heterotransplants possessed a squamous component, this provided initial evidence that keratin 7 expression would not correlate with squamous differentiation or the degree of squamous differentiation of the tumors. Furthermore, in the 6 tumor heterotransplants that did express keratin 7, there was no correlation of keratin 7 expression with areas of squamous differentiation. There was no evidence of keratin 7 overexpression in areas of squamous differentiation and, when compared to keratin 7 immunoreactive areas of the tumors, expression was reduced in intensity or absent. However, keratin 7 expression was focal in the tumors and there were also areas of low keratin 7 expression in non-squamous areas of the tumors. A very interesting trend in keratin 7 staining was that the UTCd#1 cell line that did not express keratin 7 in subcutaneous tumor heterotransplants, was the only cell line that could colonize the organs of the peritoneal cavity. The lack of keratin 7 expression noted in the subcutaneous tumors generated from the UTCd#1 cell line was also shown to extend into the cell line itself and for tumor nodules formed within the peritoneal cavity of the nude mice. All the other 6 Cd⁺² transformed cell lines were shown to express keratin 7 mRNA and protein and to have keratin 7 associated with the intermediate filaments of the cell. While only a trend for colonization of peritoneal organs, the results for keratin 7 expression do show that the phenotypic differences in the independent isolates of the Cd⁺² transformed cell do extend to the level of gene expression. Future analysis of archival specimens of human bladder cancer should show if keratin 7 expression has any relationship with the ability of TCCs to colonize local organ sites.

To the author's knowledge there have been limited studies where a single environmental toxicant, such as cadmium, has been used to generate multiple independent transformation events from a single cell line, especially those able to subsequently generate tumor heterotransplants. The results in the present study display many of the advantages and disadvantages of using an immortalized cell line and impact on how one might interpret the mechanisms underlying cadmium's ability to influence the malignant transformation and progression of the urothelial cell. On one hand, the model was effective in that all the tumors generated from the independent cell lines possessed an overall H&E histology consistent with that expected of a human urothelial carcinoma. The results also showed that tumor heterogeneity remained intact when malignant transformation and progression is elicited by exposure to cadmium. Independent cell lines were generated that possessed varying doubling times and which varied in some of the phenotypic properties of their corresponding tumor heterotransplants such as keratin 7 expression, degree of squamous differentiation, and ability to colonize internal organs. On the other hand, the interpretation of the results is also influenced by the employment of an immortal cell line. A major question that is difficult to determine is if differences in the final phenotype of each cadmium-transformed isolate was due to selection of a sub-population of cells that were present in the parent cell culture. This is always a possibility with an immortalized, but non-tumorigenic, cell line that likely possesses some degree of heterogeneity within the cell population due to the genetic instability that results from immortalization. The expression of keratin 7 illustrates this point since its expression was focal in 6 of the 7 isolate-derived tumor heterotransplants and absent in the other heterotransplant. The expression of keratin 7 was also variable in the parent cell line; leaving open the question of whether cadmium affected the selection of a few cells or an alteration of a large population of cells in the parental cell culture. The results with keratin 7 also illustrate

the value of characterizing a marker at multiple levels of examination, which in this case included real time analysis of mRNA expression, western analysis of protein expression, and localization of expression using both immunohistochemistry and intracellular imaging. These findings suggest that studies employing only a single cell line and a narrow focus to study the mechanism underlying cadmium's ability to affect tumor development or progression may be significantly influenced by the cell line chosen for use in the analysis.

Abbreviations

As ⁺³	arsenite
BCA	bicinchoninic acid
Cd ⁺²	cadmium
DMEM	Dulbecco's Modified Eagles Medium
DTT	dithiothreitol
H&E	Hematoxylin and Eosin
IP	intraperitoneal
S.C.	subcutaneous
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline Tween 20
TCC	transitional cell carcinoma

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LITERATURE CITED

1. Kellen E, Zeegers M, Hond ED, Buntinx F. Blood cadmium may be associated with bladder carcinogenesis; the Belgian case control study on bladder cancer. *Cancer Detect. Prev* 2007;31:77–82. [PubMed: 17296271]
2. Siemiatycki J, Dewar R, Nadon L, Gerin M. Occupational risk factors for bladder cancer: Results from a case control study in Montreal, Quebec, Canada. *Am. J. Epidemiol* 1994;140:1061–1080. [PubMed: 7998589]
3. Waalkes MP. Cadmium carcinogenesis in review. *J. Inorg. Biochem* 2000;79:241–244. [PubMed: 10830873]
4. Sens DA, Park S, Gurel V, Sens MA, Garrett SH, Somji S. Inorganic cadmium- and arsenite-induced malignant transformation of human bladder urothelial cells. *Toxicol. Sci* 2004;79:56–63. [PubMed: 14976345]
5. Johansson SI, Cohen SM. Epidemiology and etiology of bladder cancer. *Semin Surg. Oncol* 1997;13:291–298. [PubMed: 9259084]
6. Akdas A, Turkeri L. The impact of squamous metaplasia in transitional cell carcinoma of the bladder. *Int. J. Urol. Nephrol* 1990;23:333–336.
7. Frazier HA, Robertson JE, Dodge RK, Paulson DF. The value of pathologic factors in predicting cancer-specific survival among patients treated with radical cystectomy for transitional cell carcinoma of the bladder and prostate. *Cancer* 1993;71:3993–4001. [PubMed: 8508365]

8. Logothetis CJ, Dexeus FH, Chong C, Sella A, Ayala AG, Ro JY, Pilat S. Cisplatin, cyclophosphamide and doxorubicin chemotherapy for unresectable urothelial tumors: the MD Anderson experience. *J. Urol* 1989;141:33–37. [PubMed: 2908950]
9. Martin JE, Jenkins BJ, Zuk RJ, Blandy JP, Baithun SI. Clinical importance of squamous metaplasia in invasive transitional cell carcinoma of the bladder. *J. Clin. Pathol* 1989;42:250–253. [PubMed: 2703540]
10. Aaboe M, Marcussen N, Jensen KM-E, Thykjaer T, Dyrskjet I, Orntoft TF. Gene expression profiling of noninvasive primary urothelial tumors using microarrays. *Br. J. Cancer* 2005;93:1182–1190. [PubMed: 16265353]
11. Jiang J, Ulbright TM, Younger C, Sanchez K, Bostwick D, Kock MO, Eble JN, Cheng L. Cytokeratin 7 and cytokeratin 20 in primary urinary bladder carcinoma and matched lymph node metastasis. *Arch. Pathol. Lab. Med* 2001;125:921–923. [PubMed: 11419977]
12. Moll R, Divo M, Langhein L. The human keratins: biology and pathology. *Histochem. Cell Biol* 2008;129:705–733. [PubMed: 18461349]
13. Rossi MR, Masters JRW, Park S, Todd JH, Garrett SH, Sens MA, Somji S, Nath J, Sens DA. The immortalized UROtsa cell line as a potential cell culture model of human urothelium. *Environ. Health Perspect* 2001;109:801–808. [PubMed: 11564615]
14. San RHC, Laspia MF, Soiefer AI, Maslansky CJ, Rice JM, Williams GM. A survey of growth in soft agar and cell surface properties as markers for transformation in adult rat liver epithelial-like cell cultures. *Cancer Res* 1979;39:1026–1034. [PubMed: 427743]
15. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 1986;89:271–277. [PubMed: 3486233]
16. Flitney EW, Goldman RD. Fluorescence-based methods for studying intermediate filaments. *Methods in Cell Biology* 2004;78:297–319. [PubMed: 15646623]
17. Khanobdee K, Kolberg JB, Dunlevy JR. Nuclear and plasma membrane localization of SH3BP4 in retinal pigment epithelial cells. *Mol. Vis* 2004;10:933–942. [PubMed: 15616480]
18. Barocas DA, Clark PE. Bladder cancer. *Curr. Opin. Oncol* 2008;20:307–314. [PubMed: 18391631]
19. Pasin E, Josephson DY, Mitra AP, Cote RJ, Stein JP. Superficial bladder cancer: an update on etiology, molecular development, classification, and natural history. *Rev. Urol* 2008;10:31–43. [PubMed: 18470273]
20. Clavel J, Cordier S, Boccon-Gibod L, Hemon D. Tobacco and bladder cancer in males: Increased risk for inhalers and smokers of black tobacco. *Int. J. Cancer* 1989;44:605–610. [PubMed: 2793232]
21. Morrison AS, Buring JE, Verhoeck WG, Aoki K, Leck I, Ohno Y, Obata K. An international study of smoking and bladder cancer. *J. Urol* 1984;131:650–654. [PubMed: 6708176]
22. Vincis P, Esteve J, Hartge P, Hoover R, Silverman DT, Terracini B. Effects of timing and type of tobacco in cigarette-induced bladder cancer. *Cancer Res* 1998;48:3849–3852.
23. Murta-Nascimento C, Schmitz-Dräger BJ, Zeegers MP, Steineck G, Kogevinas M, Real FX, Malats N. Epidemiology of urinary bladder cancer: from tumor development to patient's death. *World J. Urol* 2007;25:285–295. [PubMed: 17530260]
24. Samanic C, Kogevinas M, Dosemeci M, Malats N, Real FX, Garcia-Closas M, Serra C, Carrato A, García-Closas R, Sala M, Lloreta J, Tardón A, Rothman N, Silverman DT. Smoking and bladder cancer in Spain: effects of tobacco type, timing, environmental tobacco smoke, and gender. *Cancer Epidemiol. Biomarkers Prev* 2006;15:1348–1354. [PubMed: 16835335]
25. Copes R, Clark NA, Rideout K, Palaty J, Teschke K. Uptake of cadmium from Pacific oysters (*Crassostrea gigas*) in British Columbia oyster growers. *Environ. Res* 2008;107:160–169. [PubMed: 18358470]
26. Haswell-Elkins M, McGrath V, Moore M, Satarug S, Walmbly M, Ng J. Exploring potential dietary contributions including traditional seafood and other determinants of urinary cadmium levels among indigenous women of a Torres Strait Island (Australia). *J. Expo. Sci. Environ Epidemiol* 2007;17:298–306. [PubMed: 17245391]
27. Satarug S, Moore MR. Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environ Health Perspect* 2004;112:1099–1103. [PubMed: 15238284]

28. Satarug S, Ujjin P, Vanavanitkun Y, Baker JR, Moore MR. Influence of body iron store status and cigarette smoking on cadmium body burden of healthy Thai women and men. *Toxicol. Lett* 2004;148:177–185. [PubMed: 15041068]
29. Nawrot TS, Van Hecke E, Thijs L, Richart T, Kuznetsova T, Jin Y, Vangronsveld J, Roels HA, Staessen JA. Cadmium-related mortality and long-term secular trends in the cadmium body burden of an environmentally exposed population. *Environ. Health Perspect* 2008;116:1620–1628. [PubMed: 19079711]

Figure 1. Phase contrast light microscopy of the seven Cd^{+2} transformed cell lines demonstrating epithelial morphology in all isolates. A) UTCd#1; B) UTCd#2; C)UTCd#3; D)UTCd#4; E) UTCd#5; F)UTCd#6; G)UTCd#7.

Figure 2.

Histology of tumor heterotransplants after subcutaneous injection of Cd^{+2} transformed UROtsa cell lines in nude mice. The top panels demonstrate prominent squamous features. The middle panel illustrates intermediate squamous features and the bottom panel illustrates tumors with mild features of squamous differentiation.

A) UTCd#7 with prominent squamous differentiation with stratification, intracellular bridges and prominent keratinization with keratin pearls. X200

B) UTCd#3 with prominent squamous differentiation, stratification, abundant keratin formation. X200

- C) UTCd#5 with prominent squamous differentiation. Prominent keratinization, keratin pearl formation, and stratification. X200
- D) Keratin “pearls” (arrows), concentrically laminated structures of keratin is a sign of squamous differentiation.
- E) Squamous differentiation demonstrating “stratification” of cells from small basal layer like cells (base of arrow), through larger cells with eosinophilic cytoplasm and intracellular bridges to flattened cells and keratin deposition (top of arrow).
- F) Intracellular bridges (fine network of fence-like spokes between cells, arrows) outline the epithelial cell membranes and demonstrate squamous differentiation.
- G) UTCd#1 with intermediate squamous differentiation. X200
- H) UTCd#4 with intermediate squamous differentiation. The tumor cells at the center of the tumor nests have abundant pink cytoplasm and intercellular bridges. X200
- I) UTCd#6 with intermediate squamous differentiation. X200
- J) UTCd#2 with mild squamous differentiation. Single cell keratinization is present. X200 (See K)
- K) Single cell keratinization.
- L) Clearing, vacuolization of cytoplasm (arrows) and reduced keratin formation. X400

Figure 3.

Keratin 7 immunostaining in subcutaneous and intraperitoneal tumor heterotransplants. Tissue sections were generated from tumor heterotransplants generated from each of the seven Cd^{+2} cell lines. The tissues were immunostained using an antibody to keratin 7. All micrographs are at 100x magnification. Keratin staining for subcutaneous tumor heterotransplants are shown for: A. UTCd#1; B. UTCd#2; C. UTCd#3; D. UTCd#4; E. UTCd#5; F. UTCd#6; G. UTCd#7. Figure H is a control for the UTCd#2 cell line where antibody to keratin 7 is omitted from the reaction sequence. Figure I and J show keratin 7 staining for intraperitoneal tumor nodules generated from the UTCd#1 cell line, nodules from the inner surface of the peritoneum and a nodule attached to the liver.

Figure 4.

Expression of keratin 7 in tumor heterotransplants and cadmium transformed cell lines. A and C; Real time RT-PCR analysis of expression of Keratin 7 mRNA in the tumor heterotransplants and transformed cell lines respectively. Relative mRNA levels were normalized to the change in β -actin expression as described in the materials and methods section. B and D; Western analysis of Keratin 7 protein expression in tumor heterotransplants and cell lines respectively. Integrated optical densities (IOD) for each of the Keratin 7 band is indicated. Statistical analysis consisted of ANOVA with Tukey post-hoc testing, performed by GraphPad PRISM 4. Statistically significant compared to UROtsa parent (*) and UTCd #1 (**) at the level of significance of $p < 0.05$.

Figure 5.

Immunofluorescent staining of keratin 7 in UROtsa cells. Keratin-7 immunofluorescent staining with DAPI counterstain for parental UROtsa cells (A) and the seven cadmium-transformed cell lines B) UTCd#1; C) UTCd#2; D) UTCd#3; E) UTCd#4; F) UTCd#5; G) UTCd#6; H) UTCd#7. Controls included staining using only the secondary antibody and were found to be negative for all cell lines. Keratin staining is green. Nuclei are counterstained with DAPI and are blue. Bar = 20 µm.

Figure 6. Higher magnification of keratin 7 immunofluorescent staining in the UTCd#1 (A), UTCd#3 (B) and UTCd#6(C) cell lines. The white squares in the left panel images indicate the region of high magnification shown in the right panel. Bar = 20 μ m.

TABLE 1Initial Characterization of Independently Generated Cd^{1/2} Transformed Cell Lines

Cell Line			Subcutaneous Tumors			Intraperitoneal Tumors		
Cell Line	Doubling Time (h)	Soft Agar	Subcutaneous Transplantation	Tumor Histology	Squamous Component	IP Transplantation	Tumor Histology	Squamous Component
UT Cd #1	27.8 ± 0.6	Yes	5/5	TCC	Moderate	Yes	TCC	Rare
UT Cd #2	18.8 ± 1.5 ^a	Yes	2/5	TCC	Mild	No	-	-
UT Cd #3	16.4 ± 1.8 ^a	Yes	4/5	TCC	Prominent	No	-	-
UT Cd #4	20.7 ± 1.1 ^a	Yes	2/5	TCC	Moderate	No	-	-
UT Cd #5	18.2 ± 0.8 ^a	Yes	3/5	TCC	Prominent	No	-	-
UT Cd #6	16.9 ± 1.0 ^a	Yes	2/5	TCC	Moderate	No	-	-
UT Cd #7	27.1 ± 1.3	Yes	4/5	TCC	Prominent	No	-	-
UROtsa Parent	33.2 ± 0.8	No	0/5	-	-	No	-	-

^aSignificantly different (P=0.05) from Parent UROtsa cells and UTCD#1 and #7