Metallothionein-1 and -2 Expression in Cadmium- or Arsenic-Derived Human Malignant Urothelial Cells and Tumor Heterotransplants and as a Prognostic Indicator in Human Bladder Cancer


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The goal of this study was to determine if the expression of the metallothionein (MT)-1/2 proteins might serve as a biomarker for the development of bladder cancer. A retrospective analysis of MT-1/2 staining was performed on 343 tissue sections from patients referred for the diagnosis of bladder cancer. The specimens were subdivided into six categories: benign, dysplastic, low-grade cancer, high-grade cancer with no evidence of invasion, high-grade cancer with evidence of invasion, and carcinoma in situ. There was no expression of MT-1/2 in benign lesions and low-grade cancers, a low incidence of expression in dysplastic lesions and high-grade cancers with no evidence of muscle invasion, and a significantly increased incidence of MT-1/2 in high-grade cancers that had invaded the underlying matrix. The expression of MT-1/2 varied in intensity from sample to sample and was focal in its expression. It was concluded from these findings that MT-1/2 may be a prognostic marker for cancers that are progressing to invade the underlying stroma of the bladder wall. The expression of MT-1/2 was also determined in a cell culture model of human urothelium that had been malignantly transformed by Cd²⁺ and As³⁺ and shown to be capable of tumor formation in nude mice. It was demonstrated that the expression of MT-1/2 in the tumor heterotransplants was similar to the pattern found in archival specimens of high-grade bladder cancers. The MT-1/2 staining in the heterotransplants was focal in pattern, varied in intensity, and highest in the less differentiated cells of the tumor. These findings indicate that the cell culture model may serve to help define the role of MT-1/2 expression in bladder cancer invasion.

Key Words: bladder cancer; arsenic; cadmium; metallothionein; biomarker; prognosis.

Historically, bladder cancer is the first cancer in which industrial carcinogens were found to play the major role in disease causation. There are several reports which show that the combined expression of the first and second isoforms of metallothionein (MT-1 and -2) are of prognostic significance for bladder cancer (Bahnson et al., 1991, 1994; Ioachim et al., 2001; Katoh et al., 1994; Lynn et al., 2003; Siu et al., 1998; Somji et al., 2001). The majority of these studies have focused on the relationship between MT-1/2 expression and resistance of the tumor to chemotherapy. In these studies it was demonstrated that the expression of MT-1/2 predicted the resistance of the tumor to treatment with cisplatin (Katoh et al., 1994; Siu et al., 1998), mitomycin C (Lynn et al., 2003) or combination chemotherapy with cisplatin, methotrexate, and vinblastine (Bahnson et al., 1994). The remaining studies focused more on the expression of MT-1/2 as a function of the grade of the tumor (Bahnson et al., 1991; Ioachim et al., 2001; Somji et al., 2001). While there is unanimous agreement in these studies that MT-1/2 immunoreactivity has the potential to be of prognostic significance, there is wide disagreement in the amount and distribution of MT-1/2 reactivity that occurs in human bladder tumors. Several studies report that a majority of human bladder tumors are immunoreactive for MT-1/2 (Bahnson et al., 1991, 1994; Katoh et al., 1994; Lynn et al., 2003), and two of these also report that normal urothelium can be immunoreactive for MT-1/2 (Bahnson et al., 1991; Lynn et al., 2003). Other reports have emphasized the focal nature of MT-1/2 staining in human bladder cancers (Ioachim et al., 2001; Siu et al., 1998; Somji et al., 2001). The studies of Ioachim et al. (2001) examined 63 bladder cancers and found no MT-1/2 staining in 76.2% of the cases; focal MT-1/2 staining comprising less than 10% of the tumor cells was found in 11.1%, and MT-1/2 staining comprising greater than 10% of the tumor cells in 12.7% of the cases. An examination of 118 cases of bladder cancer by Siu et al. (1998) demonstrated that 53% of the patients had tumors with less than 10% of the cells immunoreactive for MT-1/2 and the remaining tumors had greater than 10% MT-1/2-positive tumor cells. The first goal of the present study was to reexamine the immunoreactivity of MT-1/2 in benign and malignant lesions of the human bladder.

A second goal of this study was to determine if a recently developed model of human bladder cancer would mimic the
MT-1/2 expression pattern found in human bladder tumors. This model system was developed by the direct malignant transformation of an immortalized cell culture model of human urothelial cells with Cd\(^{2+}\) or As\(^{3+}\) (Rossi et al., 2001). The resulting transformants were shown to form colonies in soft agar and tumors when injected subcutaneously in immuno-compromised mice (Sens et al., 2004). The histology of the tumor heterotransplants possessed features expected of human transitional cell carcinoma.

**MATERIALS AND METHODS**

**Bladder specimens for the retrospective immunohistochemical analysis of MT-1 and -2 expression.** Tissue sections for the immunohistochemical analysis of MT-1/2 expression in the human bladder were obtained from archival paraffin blocks that originated from previously completed patient diagnostic procedures. The designation MT-1/2 is used to indicate that the E-9 antibody used in this study recognizes both the MT-1 and -2 protein isoforms of MT. The immunohistochemical expression of MT-1/2 was determined on tissue sections from 350 patients referred for diagnosis of possible transitional cell carcinoma of bladder. For analysis of MT-1/2 expression, these specimens were subdivided into six categories: benign, dysplastic, low-grade urothelial cancer, high-grade urothelial cancer with no evidence of invasion into the underlying muscular layer, high-grade urothelial cancer with evidence of invasion of the underlying muscle layer, and carcinoma in situ (CIS). Grading of the urothelial lesions was performed on hematoxylin and eosin (H&E)–stained tissue sections and utilized the World Health Organization/International Society of Urological Pathology consensus conference classification (Epstein et al., 1998).

**Tumor heterotransplant tissues for the analysis of MT-1 and -2 gene expression.** The histology of the nude mouse heterotransplants produced by subcutaneous injection of UROtsa cell lines malignantly transformed with either 1µM Cd\(^{2+}\) or As\(^{3+}\) has been described previously (Sens et al., 2004). In addition to tumor tissue harvested for histology, samples were also taken at this time for the preparation of total RNA and protein.

**Immunostaining for MT-1/2 in human and tumor heterotransplants.** Archival bladder specimens were routinely fixed in 10% neutral buffered formalin for 16–18 h. 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 Target Retrieval Solution (catalog No. S1699, Dako, Carpenteria, CA) and heated in a steamer for 20 min. The sections were allowed to cool to room temperature and immersed into Tris-buffered saline with Tween 20 for 5 min. The immunostaining was performed on a Dako Autostainer Universal Staining System. MT was localized using a monoclonal mouse anti-horse MT-1, MT-2 antibody (Dako-MT, E9) as the primary antibody. The primary antibody was localized using the DakoCytomation peroxidase-conjugated EnVision Dual Link System. Liquid diaminobenzidine (DAB) was used for visualization (DakoCytomation liquid DAB substrate chromogen system). Slides were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

The immunostaining of tumor heterotransplants was performed manually and used the identical E-9 antibody as described above. 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 specimen. Liquid DAB was used for visualization. Slides were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

The degree of MT immunoreactivity in the specimens was judged by two pathologists using two evaluation criteria. Due to the focal nature of MT-1/2 staining within urothelium and urothelial lesions, immunoreactivity was judged as the percentage of immunoreactive cells in each specimen using the following scale: 0%, 1–4%, 5–9%, 10–19%, 20–50%, and > 50% MT-1/2–positive cells. A second criterion was the intensity of the staining in MT-1/2–positive specimens, and this was judged as weak, moderate, or intense.

**Normal human bladder specimens for analysis of MT-1 and -2 mRNA expression.** Three independent samples of total RNA prepared from undiseased adult bladder were previously analyzed and shown to contain mRNA for the MT-1E, MT-1X, and MT-2A genes (Somi et al., 2001). The other active MT-1 isoforms (MT-1A, MT-1B, MT-1F, MT-1G, and MT-1H) were shown not to be expressed in these samples. These samples were used in the present study to quantify the levels of expression of the MT-1E, MT-1X, and MT-2A isoform–specific mRNAs using real-time PCR. All specimens were obtained following completion of diagnostic protocols in surgical pathology, and the protocol for tissue acquisition was approved by the Institutional Review Board for Human Research. Only samples showing no evidence of tissue degradation, as judged by histochemical examination of H&E-stained sections, were used for molecular analysis.

**Cell culture.** Stock cultures of the UROtsa cell lines malignantly transformed with either 1µM Cd\(^{2+}\) or As\(^{3+}\) were maintained in 75-cm\(^2\) tissue culture flasks in either serum or serum-free growth medium as described previously (Sens et al., 2004). Briefly, the serum-containing growth medium was Dulbecco modified Eagles medium (DMEM) containing 5% (vol/vol) fetal calf serum. The serum-free growth medium was composed of a 1:1 mixture of DMEM and Ham F-12 supplemented with selenium (5 ng/ml), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (36 µg/ml), triiodothyronine (4 ng/ml), and epidermal growth factor (10 ng/ml). Cultures in both media were incubated at 37°C in a 5% CO\(_2\)/95% air atmosphere. The cells were fed fresh growth medium every 3 days, and when confluent, the cells were subcultured at a 1:20 ratio using trypsin-EDTA.

**Real-time analysis of MT-1 and -2 isoform mRNA expression.** The measurement of MT isoform mRNA expression was assessed with real-time RT-PCR utilizing previously described MT isoform–specific primers (Mididoddi et al., 1996). Total RNA was purified from As\(^{3+}\)- and Cd\(^{2+}\)-transformed UROtsa cells growing in 9.6-cm\(^2\) culture wells in triplicate 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. Real-time PCR was performed utilizing the SYBR Green kit (Bio-Rad Laboratories) with 2 µl of cDNA, 0.2µM 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 and compared to that of a standard curve of each MT isoform gene cloned into pcDNA3.1/myc-hygro (+) and linearized with FspI. Cycling parameters consisted of denaturation at 95°C for 30 s and annealing at 65°C for 45 s which gave optimal amplification efficiency of each standard. The level of MT isoform expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) assessed by the same assay with the primer sequences being sense, TCTCTGACCTCAACAGCGACAC, and antisense, CACCCTGTGCTGTAGCCAAATTC, with a product size of 126 base pairs.

**MT protein determination.** The immunoblot protocol used for the determination of the coexpressed levels of the MT-1 and -2 proteins in cell lysates has been described previously by this laboratory (Garrett et al., 1998). The MT-1/2 proteins were detected by immunoblotting using a mouse anti-horse antibody (Dako-MT, E9) as the primary antibody.

**Statistical analysis.** All cell culture experiments were performed in triplicate, and the results are expressed as the standard error of the mean. Statistical analyses were performed using Systat software using separate variance t-tests, ANOVA with Tukey post hoc testing. Unless otherwise stated, the level of significance was 0.05.
RESULTS

Immunoreactivity of MT-1/2 in Benign, Dysplastic, and Malignant Human Urothelium

There were 350 archival patient specimens of potentially diseased urothelium available for the evaluation of MT-1/2 staining. Following H&E evaluation of the samples, seven were discarded from the study as not being primary cancers of the urothelium. The seven discarded cases were identified as a squamous cell carcinoma of the cervix, two metastatic urothelial cell cancers in lymph nodes, a metastatic urothelial cell cancer in the small intestine, a metastatic urothelial cell cancer in the brain, an adenocarcinoma of the bladder, and a prostate carcinoma. The remaining 343 samples were evaluated for MT-1/2 staining at an antibody dilution of 1:200. Of the 343 samples, 137 were evaluated as benign lesions, and of these, none were immunoreactive for MT-1/2 protein (Table 1, Fig. 1A). The examination consisted of an evaluation of the entire specimen under low (×10) and high (×40) magnification to rule out the possibility of a very low instance of focal staining. In addition, 50 cases of these benign lesions were reexamed at an antibody dilution of 1:50, and no additional staining was found for the MT-1/2 protein within the urothelium. Of the 13 dysplastic lesions available for examination, only one showed weak staining for MT-1/2, and the staining was in the lower one third of the transitional epithelium (Table 1, Fig. 1B). There were 14 cases of CIS, and two of these had focal MT-1/2 staining of weak to moderate intensity that comprised less than 10% of the cells (Table 1). There were 58 cases of low-grade urothelial cancer, and none were immunoreactive for the MT-1/2 protein (Table 1). Several of these specimens were also reexamed at an antibody dilution of 1:50, and no additional staining was found. There were 56 cases of high-grade urothelial cancer that showed no evidence of invasion into the underlying muscle layer. Of these, five specimens showed focal staining for MT-1/2 that was of moderate to strong intensity. Four of the five positive specimens had less than 10% MT-1/2–positive cells and one specimen between 10 and 20% MT-1/2–positive cells (Table 1). There were 65 cases of high-grade urothelial cancer that showed evidence of invasion into the underlying muscle layer, and 20 of these cases showed focal staining for MT-1/2 (Table 1). The MT-1/2 staining in these MT-1/2–positive specimens ranged from specimens having less than 10% of the cells MT-1/2–positive (Fig. 1C) to specimens having greater than 50% positive cells (Fig. 1D). The intensity of MT-1/2 staining also varied with some specimens having weak staining for MT-1/2 and others intense staining. The increased incidence of MT-1/2 staining in high-grade urothelial cancers with evidence of muscle invasion was significant compared to all other groups.

Immunoreactivity of MT-1/2 in Nonmalignant Stromal Cells Associated with Benign and Malignant Human Urothelium

In the course of the examination of MT-1/2 staining in the urothelium, it was also observed that there were profiles of stromal cell staining in many of the specimens. Overall, approximately 30% of the samples had some stromal cell staining for MT-1/2. In some cases, scattered positivity was seen in the stroma underlying the benign epithelium (Fig. 1E) or papillary cores of urothelial carcinoma (Fig. 1F). The presence of the MT-1/2–positive stromal cells was semiquantified as a function of the diagnostic grouping of the specimen (described above) and if the staining was associated with an identifiable structure or lesion. The identifiable lesions were the presence of: an inflammatory lesion, such as granuloma, ulcer, or necrotic area (benign urothelial ulcer, Fig. 1G); the papillary core of an urothelial carcinoma (Fig. 1F); and the invasive component of the urothelial carcinoma (Fig. 1H). A significant finding was that over 70% of the muscle-invasive areas of urothelial carcinoma had stromal cells with strong MT-1/2 immunostaining (Table 2). Furthermore, 80% of the stromal

<table>
<thead>
<tr>
<th>Disease</th>
<th>Total cases</th>
<th>Total MT-1/2 positive (%)</th>
<th>% Cells staining</th>
<th>Intensity of staining</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 10%</td>
<td>10–50%</td>
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<td>Benign</td>
<td>137</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dysplasia</td>
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<td>1 (8)</td>
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<td>1</td>
</tr>
<tr>
<td>LGC</td>
<td>58</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIS</td>
<td>14</td>
<td>2 (14)</td>
<td>2</td>
<td>0</td>
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<tr>
<td>HGC, NI</td>
<td>56</td>
<td>5 (9)</td>
<td>4</td>
<td>1</td>
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<td>HGC, I</td>
<td>65</td>
<td>20 (31)</td>
<td>11</td>
<td>5</td>
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</table>

*Low-grade carcinoma.

*High-grade carcinoma, noninvasive.

*High-grade carcinoma, invasive.
cell staining in muscle-invasive urothelial carcinoma was associated with the invasive component rather than an inflammatory lesion or papillary stromal core. The following combinations were observed: MT-1/2–negative urothelium with MT-1/2–positive stroma (Fig. 1H), MT-1/2–positive urothelium with MT-1/2–negative stroma (Fig. 1D), MT-1/2–negative urothelium and stroma (Fig. 1I), and MT-1/2–positive urothelium and stroma (not shown).

TABLE 2

<table>
<thead>
<tr>
<th>Disease</th>
<th>Total cases</th>
<th>Total MT-1/2 positive (%)</th>
<th>No associated lesion</th>
<th>Inflammatory lesion</th>
<th>Papillary core of a carcinoma</th>
<th>Invasive carcinoma</th>
<th>% Positive staining</th>
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<tr>
<td>Benign</td>
<td>137</td>
<td>28 (20)</td>
<td>10</td>
<td>18</td>
<td>N/A</td>
<td></td>
<td>56</td>
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<tr>
<td>Dysplasia</td>
<td>13</td>
<td>4 (31)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>CIS</td>
<td>14</td>
<td>2 (14)</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>LGC\a</td>
<td>58</td>
<td>11 (19)</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>HGC, NI\b</td>
<td>56</td>
<td>15 (27)</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>N/A</td>
<td>27</td>
</tr>
<tr>
<td>HGC, I\c</td>
<td>65</td>
<td>46 (71)</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>36</td>
<td>7</td>
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</table>

\aLow-grade carcinoma.

\bHigh-grade carcinoma, noninvasive.

\cHigh-grade carcinoma, invasive.
Immunoreactivity of MT-1/2 in Tumors Derived from Heterotransplanted \textit{As}^{3+}- and \textit{Cd}^{2+}-transformed \textit{UROtsa} Cells

In a previous study, tumor heterotransplants were generated from an immortalized urothelial cell line (UROtsa) that had been transformed by exposure to \textit{Cd}^{2+} and \textit{As}^{3+} (Sens et al., 2004). In total, four malignantly transformed cell lines were isolated that were capable of generating tumor heterotransplants in nude mice: URO-ASSC for cells transformed with \textit{As}^{3+} on serum-containing growth medium, URO-ASSF for cells transformed with \textit{As}^{3+} on serum-free growth medium, URO-CDS for cells transformed with \textit{Cd}^{2+} on serum-containing growth medium, and URO-CDSF for cells transformed with \textit{Cd}^{2+} on serum-free growth medium. This previous study detailed the histology of the respective heterotransplants generated from these four cell lines, and tissue sections from these tissue blocks were used in the present study to determine the immunostaining of the MT-1/2 proteins. The results disclosed that the tumors derived from all four cell lines displayed focal staining for MT-1/2 that varied considerably in intensity within each tumor (Figs. 2A–2D). The one consistent feature that distinguished MT-1/2 staining among the tumors was that the more undifferentiated areas showed a trend for enhanced MT-1/2 staining while more differentiated areas (asterisks, Fig. 2C) of the tumors were negative or had very reduced levels of MT-1/2 staining. The tumors themselves are each very heterogeneous as regards differentiation, and this alone prevented distinguishing one tumor from another based on a pattern of MT-1/2 reactivity. Overall, each tumor displayed focal staining for the MT-1/2 protein and staining was greatest in areas of least differentiation of the tumor cells. A second observation was that there were profiles of stromal staining for MT-1/2 in each of the four tumors (Figs. 2E and 2F). There was no obvious difference in the pattern of stromal staining among the four tumors, and stromal staining was not associated with specific areas of tumor differentiation.

Urothelial Cell Expression of MT-1 and -2 mRNAs and Protein

Real-time PCR was used to determine the levels of expression of the MT-1E, MT-1X, and MT-2 mRNAs relative to that of the G3PDH housekeeping gene in total RNA preparations from three samples of normal human bladder tissue. The mRNA expression level of the MT-1E gene was found to be 1.29 ± 0.68 transcripts, that of the MT-1X gene 0.21 ± 0.09 transcripts, and that of the MT-2A gene 0.15 ± 0.06 transcripts. The expression of the MT-1E, MT-1X, and MT-2A mRNAs and the MT-1/2 protein levels was also determined for the parental UROtsa cells and for the cells after malignant transformation with \textit{Cd}^{2+} and \textit{As}^{3+}. The determinations were performed on cells under the following conditions: the parental UROtsa cell line grown on serum-containing and serum-free growth medium; the UROtsa cells that had been exposed to either 1\mu M \textit{Cd}^{2+} or 1\mu M \textit{As}^{3+} continuously during the time course that resulted in the ability of the cells to proliferate in soft agar and form tumors in nude mice; and UROtsa cells that were known to be capable of tumor formation that had not been exposed to either \textit{Cd}^{2+} or \textit{As}^{3+} for an additional five passages at a 1:20 split ratio. The level of the MT-1/2 protein in the parental UROtsa cells was 1.6 ± 0.2 and 1.3 ± 0.2 for cells grown in serum-free and serum-containing medium, respectively (Fig. 3A).

The level of MT-1/2 protein in \textit{Cd}^{2+}-transformed UROtsa cells grown in the continued presence of 1\mu M \textit{Cd}^{2+} was significantly increased compared to both parental cells and \textit{Cd}^{2+}-transformed cells passaged in the absence of \textit{Cd}^{2+} (Fig. 3A).

The level of MT-1E mRNA in parental UROtsa cells was between 0.25 and 0.50 transcripts per number of G3PDH regardless of growth medium composition (Fig. 3B). There was no significant difference in MT-1E mRNA level compared to control for \textit{Cd}^{2+}-transformed UROtsa cells maintained in serum-containing growth medium in the presence or absence of
Cd\textsuperscript{2+}. This was also true for Cd\textsuperscript{2+}-transformed cells in serum-free growth medium maintained in the absence of Cd\textsuperscript{2+}. There was a significant increase in MT-1E mRNA level for Cd\textsuperscript{2+}-transformed cells grown in serum-free medium in the continued presence of Cd\textsuperscript{2+} when compared to parental cells or the other Cd\textsuperscript{2+}-transformed cells. The level of MT-1X mRNA in parental UROtsa cells was approximately 0.03 transcripts per G3PDH regardless of growth medium composition (Fig. 3C). The level of MT-1X mRNA was elevated compared to control in all four groups of Cd\textsuperscript{2+}-transformed cells (Fig. 3C). In addition, the Cd\textsuperscript{2+}-transformed cells grown on serum-free medium in the continued presence of Cd\textsuperscript{2+} also had significantly elevated levels of MT-1X mRNA compared to all other groups of Cd\textsuperscript{2+}-transformed cells. The level of MT-2A mRNA in parental UROtsa cells was approximately 0.05 transcripts per G3PDH regardless of growth medium composition (Fig. 3D). There was no significant difference in the expression of MT-2A mRNA compared to control for Cd\textsuperscript{2+}-transformed UROtsa cells grown in the presence of Cd\textsuperscript{2+} in both serum-containing and serum-free growth media.

The UROtsa cells that were transformed by As\textsuperscript{3+} in serum-free medium and grown in the absence of As\textsuperscript{3+} for five passages had significantly increased levels of MT-1/2 protein compared to both parental cell lines and all other UROtsa cell lines transformed by As\textsuperscript{3+} (Fig. 3A). All other As\textsuperscript{3+}-transformed cell lines had MT-1/2 protein levels similar to those found in parental cells.

There was no significant difference in MT-1E mRNA levels compared to control for As\textsuperscript{3+}-transformed UROtsa cells maintained in serum-containing growth medium in the presence or absence of As\textsuperscript{3+} (Fig. 3B). This was also true for As\textsuperscript{3+}-transformed cells in serum-free growth medium maintained in the absence of As\textsuperscript{3+}. There was a significant increase in MT-1E mRNA level for As\textsuperscript{3+}-transformed cells grown in serum-free medium in the continued presence of As\textsuperscript{3+} when compared to parental cells or the other As\textsuperscript{3+}-transformed cells. The level of MT-1X mRNA was significantly elevated for UROtsa cells transformed by As\textsuperscript{3+} in serum-free medium and grown in the continued presence of As\textsuperscript{3+} when compared to parental cells.
or other As³⁺-transformed cell lines (Fig. 3C). The other As³⁺-transformed cell lines had MT-1X mRNA levels similar to those found in parental cells. There was no significant difference in the level of expression of MT-2A mRNA for As³⁺-transformed cell lines when compared to parental cells or to one another (Fig. 3D).

Expression of MT-1 and -2 mRNAs and Protein in Tumor Heterotransplants

The expression of the MT-1E, MT-1X, and MT-2A mRNAs and the MT-1/2 protein levels was also determined on tumor heterotransplants that formed following the injection of the UROtsa cells that were malignantly transformed with Cd²⁺ and As³⁺ (Table 3). The tumors that resulted from UROtsa cells that were grown and transformed by Cd²⁺ in serum-containing medium were shown to express levels of MT-1/2 protein that were significantly higher than those generated from UROtsa cells grown and transformed by Cd²⁺ in serum-free growth medium (Table 3). A similar finding was found for the tumor heterotransplants formed from As³⁺-transformed cells, with cells transformed in serum-containing growth medium having a significant elevation of MT-1/2 protein levels (Table 3). There was no significant difference between the MT-1/2 protein levels in tumors that arose from UROtsa cells grown and transformed by Cd²⁺ and As³⁺ in serum-containing growth medium. The same was true for MT-1/2 protein levels in tumors arising from UROtsa cells grown and transformed by Cd²⁺ and As³⁺ in serum-free growth medium (Table 3).

All the tumor heterotransplants were shown to express MT-2A, MT-1E, and MT-1X mRNAs (Table 3). There was no significant difference in the expression of MT-2A and MT-1X mRNAs among any of the four sets of tumor heterotransplants. The expression of MT-1E mRNA was similar among three of the four sets of tumor heterotransplants, with only the tumors derived from UROtsa cells grown and transformed by Cd²⁺ in serum-free growth medium having significantly increased levels of expression (Table 3).

<table>
<thead>
<tr>
<th>Transformation condition</th>
<th>MT-1/2 protein⁵</th>
<th>MT-2A⁶</th>
<th>MT-1E⁶</th>
<th>MT-1X⁶</th>
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<tr>
<td>Cd²⁺-serum</td>
<td>9.52⁺⁺⁺</td>
<td>0.042</td>
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<td>0.703</td>
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<tr>
<td>Cd²⁺-serum free</td>
<td>4.00</td>
<td>0.102</td>
<td>5.46⁺⁺⁺</td>
<td>1.70</td>
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<tr>
<td>As³⁺-serum</td>
<td>11.50⁺⁺⁺</td>
<td>0.031</td>
<td>2.11</td>
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</tr>
<tr>
<td>As³⁺-serum free</td>
<td>5.90</td>
<td>0.026</td>
<td>1.60</td>
<td>0.56</td>
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</table>

⁵Number of tumor samples.
⁶MT protein expressed as nanograms of MT per microgram of total protein.

DISCUSSION

The overall goal of this study was to determine if the expression of the MT-1/2 protein might serve as a biomarker for the development of bladder cancer. This expectation was based on several factors. There is an extensive literature that defines the role of the MT-1 and -2 isoforms as mediators of environmental insult (Andrews, 2000; Cousins, 1983; Goering and Klaassen, 1983; Hamer, 1986; Kägi, 1993; Kägi and Hunziker, 1989). Bladder cancer is also the first cancer in which industrial carcinogens were found to play the major role in disease causation. This was first observed by Rehn (1895) when it was demonstrated between exposure to aromatic amines and development of bladder cancer in factory workers and extends to the present through associations with smoking (Clavel et al., 1989; Morrison et al., 1984) and, more recently, with arsenic (Steinmaus et al., 2000) and cadmium (Siemiatycki et al., 1994; Waalkes, 2000). As an environmentally induced cancer, it is preceded by almost a century by correlations of snuff in relation to nasal cancers and scrotal cancer in chimney sweeps. Furthermore, as detailed in the introduction, the positive staining of MT-1/2 in bladder tumors has been associated with resistance to several forms of chemotherapy. From these combined observations, one could predict that the expression of the MT-1/2 isofrom proteins might be associated with the development of bladder cancer.

The results of the present study provide strong evidence that the expression of the MT-1/2 protein is not a biomarker for the development of bladder cancer. In the present analysis, 137 independent archival specimens of human bladder were examined for the expression of the MT-1/2 proteins by staining with the E-9 antibody, and no urothelial cells were found to be immunoreactive for the MT-1/2 protein. These archival specimens were obtained from suspected bladder cancer patients, and at least some would be expected to express MT-1/2, if expression was to be a biomarker for cancer development. This was further reinforced by the finding that none of the 58 cases of low-grade bladder cancer expressed MT-1/2 protein.
Similarly, only one of 13 dysplastic lesions, two of 14 cases of CIS, and five of 56 cases of noninvasive high-grade bladder cancers were found to stain for the MT-1/2 protein. The results of the present study also reinforced the findings of earlier studies that indicated a limited occurrence of MT-1/2 staining in bladder cancer and, when present, focal staining (Ioachim et al., 2001; Siu et al., 1998; Somji et al., 2001). This is in contrast to other earlier studies that reported that a majority of human bladder tumors are immunoreactive for MT-1/2 (Bahnson et al., 1991, 1994; Katoh et al., 1994; Lynn et al., 2003). The present studies also reinforce the earlier observations that indicated no staining for MT-1/2 in normal or benign human bladder (Ioachim et al., 2001; Siu et al., 1998; Somji et al., 2001). Thus, the present study shows that MT-1/2 protein expression is not increased during the early development of bladder cancer; therefore, MT-1/2 expression would not be a candidate biomarker indicating the development of bladder cancer.

In contrast, the present study does suggest that the increased expression of MT-1/2 protein might become a candidate as a prognostic marker associated with disease progression in late stages of bladder cancer. This is based on the finding that 20 of 65 (30.7%) high-grade tumors with invasion of the underlying muscle stained positive for MT-1/2 protein. This is in contrast to MT-1/2 staining in only five of 56 (8.9%) high-grade tumors showing no evidence of invasion into the underlying muscle. The conclusion from this observation is that there is a significant association of MT-1/2 staining with bladder cancers that invade the underlying muscle. An analysis of the patient outcome associated with these specimens is underway to determine if MT-1/2 staining correlates with this important prognostic parameter. From these data, the next logical question one would like to address is if metastatic lesions arising from these or related bladder cancers overexpress the MT-1/2 protein. Unfortunately, such samples for this type of analysis are extremely rare. This is due to the fact that surgical resection of metastatic sites is rare, and thus, specimens for analysis are unavailable for study. It is also rare that tissue is available postmortem due to the low rate of such examinations on cancer patients. Thus, within the limitations imposed by the use of human samples, the staining of MT-1/2 appears to be a marker associated with bladder cancers that are invading the underlying muscle.

The analysis of the tumor heterotransplants produced by injection of UROtsa cells malignant transformed with either Cd\(^{2+}\) or As\(^{3+}\) should complement and extend the above findings that employ human specimens. The initial characterization of these subcutaneous heterotransplants showed tumor histology consistent with that of human transitional cell carcinoma (Sens et al., 2004). The analysis of MT-1/2 expression in these heterotransplants was consistent with the findings noted in human bladder cancer. Similar to patient specimens, the staining of MT-1/2 in each of the heterotransplants was focal in nature with areas of negative, weak, moderate, and strong staining of MT-1/2. Additionally, the analysis of the staining in the heterotransplants showed that MT-1/2 staining was localized to cells in the undifferentiated areas of the tumors, with reduced or no expression in areas containing more differentiated cells. The association of MT-1/2 staining with undifferentiated tumor cells would be consistent with staining being associated with more aggressive areas of a tumor. The tumor heterotransplants were all also shown to express MT-1/2 protein by immunoblot and MT-1E, MT-1X, and MT-2A mRNAs using real-time PCR. The findings with the subcutaneous heterotransplants suggest that cell lines could be isolated from these tumors that do and do not express the MT-1/2 protein. It would then be possible to inject such tumors through the tail vein and left ventricle of nude mice to determine the metastatic potential and subsequent expression of MT-1/2. Similarly, the original transformed cell cultures can also be injected to determine if metastatic colonies express the MT-1/2 protein. Implantations under the renal capsule and in the bladder are related methods to study the differentiation, invasion, and spread of the tumor cells as it relates to the expression of MT-1/2. Such studies will also provide additional information on the characteristics of urothelial cells malignantly transformed by two major environmental toxicants. Thus, the heterotransplants should provide a means to complement the human analysis that suggests a possible role of MT-1/2 expression in bladder cancer invasion and metastasis.

An unexpected observation in the present study was that some stromal cells stained positive for the MT-1/2 protein in the human archival specimens. Instances of stromal staining for MT-1/2 were the most frequent in high-grade tumors that had invaded the underlying matrix of the bladder. However, all tumor classes as well as benign lesions showed some specimens with staining of stromal cells. In benign lesions these were associated predominantly with areas of necrosis or inflammation, possibly suggesting a response associated with wound healing. In the tumor specimens, the majority of the MT-1/2–positive stromal cells were found in areas of the tumor where new stroma had formed in association with the tumor cells and not in established areas of the underlying matrix of the bladder wall. The formation of new stroma at sites of active tumor invasion is a common event in human malignancy and the term “stromatogenesis” has been used to describe this process (Sivridis et al., 2004, 2005). What is new in the present study is that these stromal cells, or a subset thereof, can be identified through their immunoreactivity for MT-1/2. To the authors’ knowledge, the increased association of new stroma with invading bladder cancer is also a new observation for this disease. Stromal staining for MT-1/2 was also noted in association with the tumors formed by injection of the Cd\(^{2+}\)- and As\(^{3+}\)-transformed tumor cells subcutaneously in the nude mice. This would indicate that the heterotransplanted cells stimulate the recruitment of mouse stromal cells that express the MT-1/2 protein to the site of tumor growth.

A question that cannot be answered by this study is if the increased MT-1/2 staining has any causal relationship with invasion of the underlying stroma and/or metastasis. The role
of MT-1/2 as a major zinc-binding protein, whose overexpression might perturb cellular zinc status, would certainly provide an opportunity for its indirect participation in many processes associated with tumor invasion and metastasis. For example, the metalloproteinases are zinc-dependent endopeptidases collectively capable of degrading essentially all components of the extracellular matrix (Vihinen and Kahari, 2002). However, the zinc proteome is extensive, with genes encoding only zinc finger domains exceeding 3% of the identified human genes (Clarke and Berg, 1998; Maret, 2001). The combination of comparing archival human specimens with a cell culture–based model that retains in situ properties and is capable of heterotransplantation should provide a platform to study the role of zinc and MT in tumor invasion and metastasis.

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