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Advances in Brief

Metallothionein Localization and Cisplatin Resistance in Human Hormone-independent Prostatic Tumor Cell Lines[1]

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Abstract

Metallothioneins (MT) are major cysteine-rich proteins with poorly characterized functions. We have examined the MT amount, isotype expression, and subcellular distribution in 4 human hormone-independent prostatic carcinoma cell lines. Both PC-3 and DU-145 cells were thiol-rich cells with similar MT and glutathione levels, while HPC36M and PC-3 MA2 were thiol-poor cells with lower MT and glutathione levels. All 4 prostatic cell lines expressed the MTIIA isoform at a basal level; DU-145 cells also constitutively expressed MTIE mRNA. Using antibodies for both total MT and MTIIA, we defined MT to cytoplasmic and nuclear domains in PC-3 cells, to perinuclear and nuclear domains in HPC36M cells, and to prominent nonnucleolar nuclear domains in DU-145 and PC-3 MA2 cells. These results indicate that the subcellular distribution is cell type specific and not reflective of the total MT content or MT isoform. Resistance to cadmium in all 4 cell lines was correlated with total MT levels, while resistance to the anticancer agent cisplatin correlated best with nuclear MT content. We suggest that the subcellular localization of MT is functionally important in cellular protection against the anticancer agent cisplatin in human prostatic cancer cells.

Introduction

MTs[4] are low molecular weight, thiol-rich cellular proteins constitutively expressed in a wide variety of organisms from yeast to humans. MT selectively binds heavy metal ions such as the essential trace elements zinc and copper, as well as the potentially toxic elements cadmium and mercury. The full biological functions of these proteins are not clearly resolved but undoubtedly include important roles in metal homeostasis and detoxification (1). At least 7 distinct human MT isoforms have been identified, although, with the exception of MTIII, no unique biochemical or biophysical property has been observed. MTs are readily inducible by metals, hormones, and cytokines. Despite its relatively small mass (6 kDa), recent studies suggest that MT can localize in the cytoplasm and nucleus of normal and malignant cells. Although the functional significance and mechanisms for this sequestration are not known, it has been suggested that nuclear localization is promoted by increased intracellular MT levels and by rapid cellular proliferation (2) or movement into S phase (3). Several studies suggest that expression of MT affords protection against the carcinogenic effects of cadmium or anticancer agents (4, 5) and the cytotoxicity of electrophilic mutagens, such as *N*-methyl-*N'*-nitrosoguanidine and *N*-nitro-*N*-methylurea, and anticancer agents, such as chlorambucil, melphalan, and cisplatin (6, 7). Protection, however, has not always been seen when MT is overexpressed (8, 9), which could reflect differences in the MT isoform composition after induction or the subcellular distribution of MT. In the current study we have examined the thiol content, MT isotype expression, and subcellular distribution in 4 human hormone-independent prostatic carcinomas that differ in their sensitivity to cadmium and cisplatin.

Materials and Methods

Cell Lines, Culturing Condition, and Chemicals. DU-145 and PC-3 cells were obtained from the American Type Culture Collection (Bethesda, MD). PC-3 MA2, subclone from PC-3, and HPC36M were kindly provided by Dr. Brigit T. Hill (Centre de Recherche Pierre Fabre, Castres, France). Cells were grown as monolayer cultures in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 IU/ml), and streptomycin (100 μg/ml). All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C and were routinely found to be free of *Mycoplasma*. Cisplatin was obtained from Bristol-Myers Squibb (Wallingford, CT). Unless noted otherwise, all other reagents were obtained from Sigma (St. Louis, MO).

Quantification of MT and GSH. The cells were plated at 1×10^6/10-cm tissue culture plate (Falcon, Lincoln Park, NJ). After incubation for 48 h, cells were harvested by treatment with 0.1% trypsin/0.44 mM EDTA in Puck's saline A and lysed in 10 mM Tris buffer (pH 8) with 1 μg/ml $CdCl_2$ by three freeze-thawing cycles followed by a brief sonication. The lysate was centrifuged at 10,000 × *g* for 10 min, and the supernatant was used for the ^{109}Cd binding and GSH assay. Total cellular MT was quantified by the binding of carrier-free $^{109}CdCl_2$ (specific activity, 136 μCi/μg cadmium; Amersham, Arlington Heights, IL) as reported previously (10); total cellular GSH was measured by the glutathione reductase 5,5'-dithiobis(2-nitrobenzoic acid) assay of Tietze (11). Cell protein concentration was measured by the Bradford assay (12).

RNA Isolation and Northern Blot Analysis. Total cellular RNA was extracted by acid guanidinium isothiocyanate-phenol-chloroform extraction (13), a isoform expression determined by Northern blotting with hMT isoform-specific oligomer probes, as described previously (13). Hybridization were performed as described by Yang *et al.* (13). The autoradiograms were generated using a Molecular Dynamics PhosphorImager, and the band intensity was quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA). All blots were stripped and rehybridized with a 28S rRNA 40-mer probe (Oncogene Science, New York, NY) to ensure integrity of the RNA samples and to confirm that equal amounts of RNA had been loaded onto each lane.

Immunocytochemical Localization of MT. Cells were plated at a density of approximately $1 \times 10^4/cm^2$ on a multichamber slide (Nunc, Inc., Naperville, IL) and incubated for 48 h at 37°C. The medium was removed and cells were washed three times with PBS (pH 7.4). Cells were fixed and permeabilized by incubation in 2% paraformaldehyde/0.1% Triton X-100 for 15 min at room temperature. The cells were washed with PBS, and nonspecific antibody binding was blocked by two washes for 5 min in PBS containing 0.5% BSA and 0.15% glycine, followed by incubation for 30 min at 25°C with the same solution supplemented with 5% normal goat serum. Cells were incubated at 25°C for 2 h with approximately 5 μg/ml (specific IgG concentration) of a previously described antisera (10) [preimmune rabbit serum, affinity-purified rabbit anti-MT serum (M2), which recognizes all human MT isoforms, or an epitope specific anti-hMTIIA rabbit antisera (B2)]. After three washes with PBS, the primary antibody was revealed with goat tetramethylrhodamine

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[4] The abbreviations used are: MT, metallothionein; GSH, glutathione; M2, anti-MT antiserum; B2, anti-human MTIIa antiserum; hMT, human metallothionein.

Table 1 *Metallothionein and glutathione content in human hormone-independent prostatic tumor cell lines*

Cell type	MT (μg/mg protein)	GSH (nmol/mg protein)
DU-145	1.40 ± 0.15[a]	128.0 ± 8.6
PC-3	1.48 ± 0.05	98.0 ± 18.6
PC-3 MA2	0.49 ± 0.03	73.7 ± 2.5
HPC36M	0.42 ± 0.06	69.4 ± 5.8

[a] Mean ± SE for 3 different determinations.

isothiocyanate-conjugated anti-rabbit secondary antibody. The cells were incubated for 1 h and washed three times with PBS. The cells were mounted in Gelvatol (Monsanto, St. Louis, MO) and immediately examined with a Molecular Dynamics Microprobe 2001 laser scanning microscope.

Colony-forming Assay. Cells were plated at 200 cells/60-mm dish. After a 24-h incubation at 37°C, cells were washed once with PBS at room temperature and reincubated in RPMI 1640 without serum; 0.1–100 μM cisplatin or 0.01–100 μM $CdCl_2$ was added and cells were incubated at 37°C for 1 h. Following drug exposure, cells were washed twice with PBS at room temperature and reincubated in complete medium. Colonies were stained with crystal violet, and the number of colonies containing more than 200 cells was counted after a 7-day incubation at 37°C. Untreated controls from each culture were examined in parallel; plating efficiency for the untreated cells was >70%.

Statistical Analysis. All data were analyzed for statistical significance by the Student *t* test. $P < 0.05$ was considered to indicate differences that were statistically significant.

Results

The basal MT concentrations in DU-145 and PC-3 were similar [1.40 ± 0.15 (SE) and 1.48 ± 0.05 μg MT/mg protein, respectively] and were significantly greater than in PC-3 MA2 and HPC36M cells (0.49 ± 0.03 and 0.42 ± 0.05, respectively) (Table 1). Cells with higher levels of MT also appeared to have higher levels of GSH (Table 1). The GSH levels in DU-145 cells are similar to those published previously (14). We next examined expression of MT isoforms in DU-145, PC-3, PC-3 MA2, and HPC36M cell lines by Northern blot analysis and found that the hMTIIA levels in DU-145 and PC-3 were almost identical and greater than the steady state levels in PC-3 MA2 and HPC36M cells. Only DU-145 cells expressed basal hMTIE; no detectable hMTIA, hMTIB, hMTIF, and hMTIG expressions were seen in any cell types. The subcellular distribution of total MT was studied by immunofluorescence using an antiserum recognizing either all MT isoforms (M2) or hMTIIA (B2). The immunoflu-

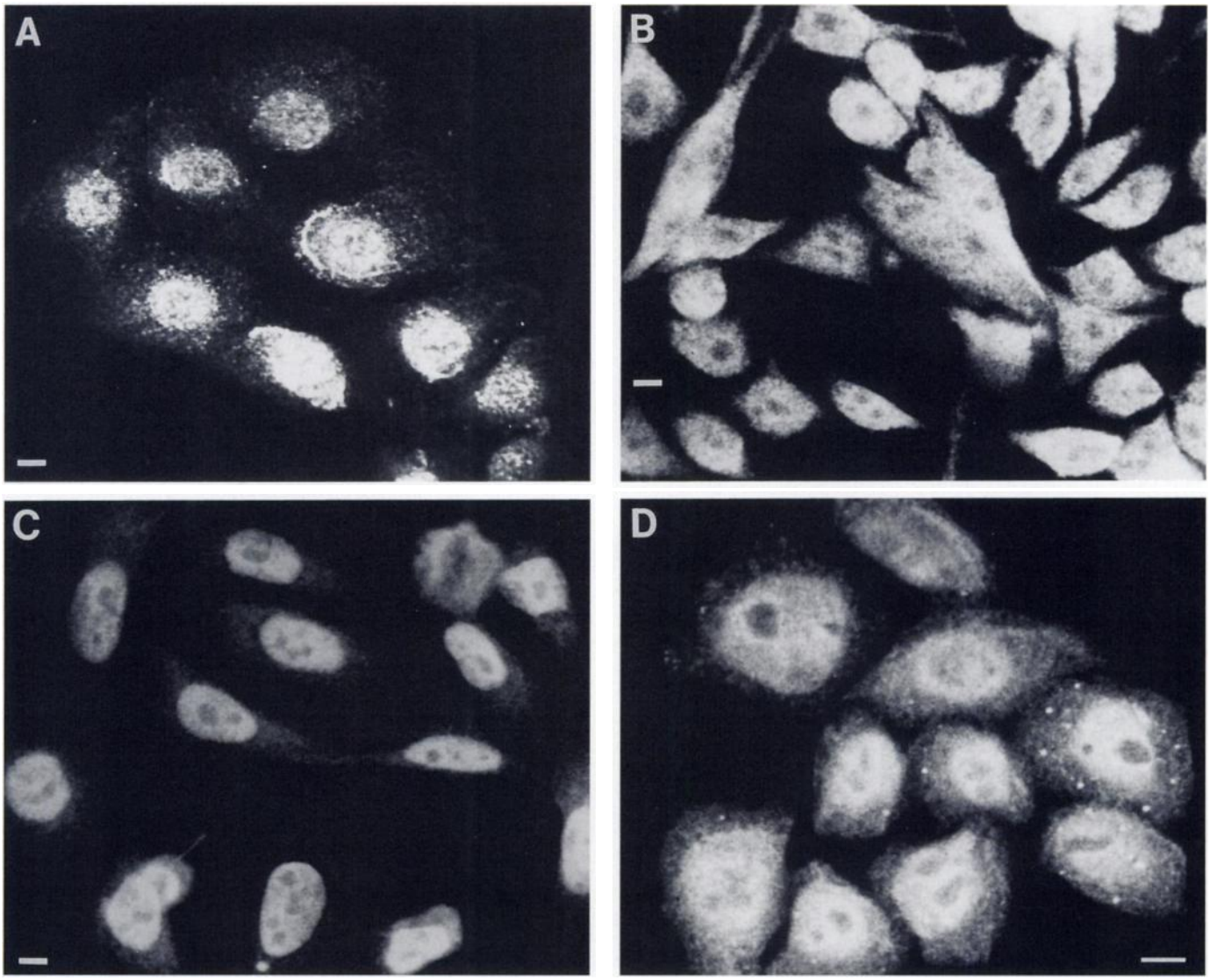

Fig. 1. Subcellular localization of MT in four human hormone-independent tumor cell lines. Cells were incubated for 48 h. After fixation and incubation with an affinity-purified M2 (1:20) and a goat tetramethylrhodamine isothiocyanate-conjugated secondary antiserum (1:100), the cells were viewed by confocal microscopy. *A*, DU-145 cells; *B*, PC-3 cells; *C*, PC-3 MA2 cells; *D*, HPC36M cells. The optical sections shown are from the region of cells containing the maximum nuclear dimension. *Bar*, 5 μm.

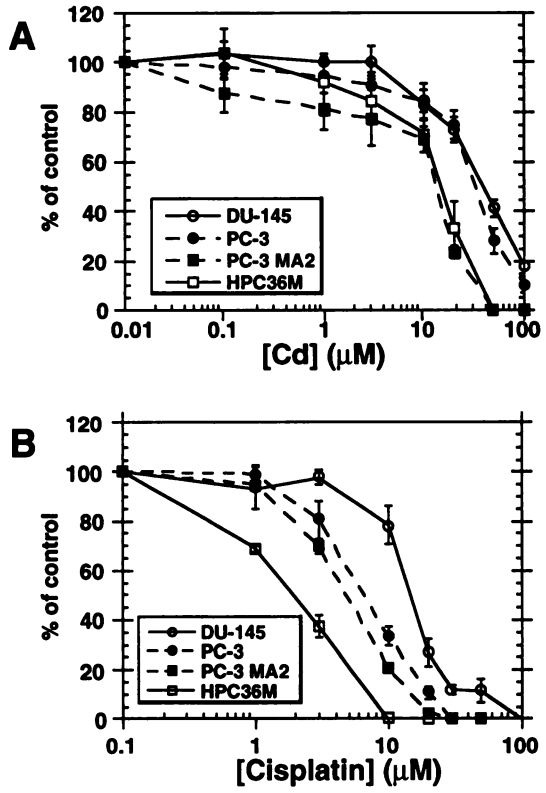

Fig. 2. Cytoprotection against cadmium and cisplatin in four human hormone-independent prostatic cell lines. Cells were incubated for 24 h and then treated for 1 h with various concentrations of cadmium (*A*) and cisplatin (*B*). $n = 3$; *bars*, SEM (unless it is smaller than the size of symbol).

orescence with M2 and B2 showed almost exclusive nuclear localization of MT in DU-145 and PC-3 MA2 cells and both cytoplasmic and nuclear localization of MT in PC-3 and HPC36M cells (Fig. 1).

To evaluate the potential functional importance of MT subcellular localization, we next measured the survival of these cell lines after exposure to various concentrations of $CdCl_2$ and cisplatin. Colony survival curves obtained after a 1-h $CdCl_2$ exposure demonstrated a concentration-dependent loss of viability of these 4 cell lines (Fig. 2*A*). The cadmium sensitivity of the thiol-rich cell lines, DU-145 and PC-3, were similar between each other but markedly less than that of the thiol-poor cell lines, PC-3 MA2 and HPC36M. The $CdCl_2$ 50% inhibitory concentrations for DU-145, PC-3, PC-3 MA2, and HPC36M were 41.7, 35.3, 14.1, and 15.6 μM, respectively. These 4 cell lines also demonstrated a concentration-dependent loss of colony-forming ability with a 1-h cisplatin exposure (Fig. 2*B*). The two thiol-rich cell lines were more resistant to cisplatin than were the two thiol-poor cell lines. Interestingly, DU-145 cells, which showed prominent nuclear MT, were significantly more resistant to cisplatin than were PC-3 cells, which had a more homogenous MT staining. A similar profile was observed for the thiol-poor cell lines with PC-3 MA2 (nuclear MT) being more resistant than HPC36M (nuclear and cytoplasmic MT); the cisplatin 50% inhibitory concentrations for DU-145, PC-3, PC-3 MA2, and HPC36M cells were 14.0, 6.5, 4.0, and 1.8 μM, respectively.

Discussion

Nonprotein and protein thiols, such as MT, appear to have an important role in protecting cells against electrophilic agents, including heavy metals, mutagens, and some antineoplastic drugs. The precise mechanism of action of MT in protecting cells against these toxins, however, has not been established, and there is some controversy about the importance of MT in anticancer drug resistance (8, 9). In the current studies, we have used four previously untreated cultured prostate cell lines as models to investigate the relationship between MT levels and cisplatin or heavy metal sensitivity. Prostatic cells are particularly relevant because little is known about the MT levels in these cells, although low MT levels have been reported in normal rat prostate (15), and cadmium has been shown to cause prostatic tumors in rodents (16). Moreover, hormone-independent prostatic tumors are essentially refractory to conventional chemotherapy.

Although these four prostatic tumor cell lines were derived from the same histological tumor type and have similar doubling times in culture, they could be separated into thiol-rich and thiol-poor cells (Table 1). These thiol-rich and thiol-poor cells exhibit different sensitivities to cadmium and cisplatin. While we recognize that cellular sensitivity to cisplatin is multifactored, it is both interesting and provocative that these 4 cell lines exhibit differences in MT subcellular distribution. Using both an antiserum that recognizes the major human isoform, MTIIA, and an antiserum that recognizes all isoforms, PC-3 and HPC36M cells displayed both cytoplasmic and nuclear MT while DU-145 and PC-3 MA2 had a prominent nonnucleolar nuclear MT phenotype. The molecular basis for this difference in MT subcellular distribution and its functional significance are not yet known. Nuclear MT was reported in hepatic and renal cells from control and $CdCl_2$-injected rats (17) but these are the only cultured nonmalignant cells that have been reported to have nuclear MT. Nuclear MT has been seen previously in cultured malignant cell lines (10). Human astrocytoma cells treated with interleukin-1, cadmium, and zinc express nuclear MT (18), and hepatocytes stimulated with insulin and epidermal growth factor caused nuclear translocation of MT during early S phase (3). Thus, nuclear MT may assume a functional significance in heavy metal detoxification or cellular proliferation. Pretreatment of mice with MT-inducing metals can protect target organs, such as the kidney, from cisplatin toxicity (19). Our results demonstrate that factors other than total MT content or the MT isotype may determine its distribution and that malignant cells of a distinct pathological class can exhibit different MT distributions. MT has no known nuclear localization signal. Thus, the nuclear sequestration of MT may reflect an interaction of MT with nuclear constituents or the existence of a nuclear MT transport process.

Previous studies suggest total intracellular MT levels are insufficient to account fully for cellular resistance to antineoplastic and mutagenic agents because protection has not always been seen after MT overexpression (8, 9). Because many antineoplastic agents and mutagens have DNA as their target, a nuclear concentration of MT might be critical to its protective function. Studies (20) have demonstrated DU-145 cells, which have nuclear MT, are approximately 4-fold more resistant to melphalan than PC-3 cells, which have a more diffuse MT distribution. Recently, it was noted that nuclear MT confers protection to DNA from hydroxyl radical attack (21). Thus, nuclear MT could have an important role not only for anticancer drug detoxification but also for free radical scavenging. Our current study suggests that MT subcellular localization should be examined in more detail. It would also be interesting to determine if the subcellular distribution of GSH is altered in cells with more nuclear MT, as MT has been shown to interact with this nonprotein thiol (22).

In conclusion, we found that human hormone-independent prostatic cell lines can exhibit different patterns in the subcellular distribution of MT. DU-145 and PC-3 MA2 cells which had primarily nuclear MT were more resistant to cisplatin compared to PC-3 and HPC36M cells, respectively, which had more diffuse cytoplasmic, perinuclear, and nuclear MT. We propose the nuclear localization of MT is an

important determinant for its protective function against electrophilic antitumor agents such as cisplatin.

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