Exposure of Human Proximal Tubule Cells to Cd^{2+}, Zn^{2+}, and Cu^{2+} Induces Metallothionein Protein Accumulation but not Metallothionein Isoform 2 mRNA

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The organization of the human metallothionein (MT) gene family is more complex than the commonly used mouse and rat models. The human MTs are encoded by a family of genes consisting of 10 functional and 7 nonfunctional MT isoforms. One objective of this study was to determine if the accumulation of MT protein in cultures of human proximal tubule (HPT) cells exposed to metals is similar to that expected from the knowledge base obtained from rodent models. To accomplish this objective, HPT cells were exposed to both lethal and sublethal concentrations of Cd^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}, Hg^{2+}, and Pb^{2+} and MT protein levels were determined. The results were in general agreement with animal model studies, although there were some exceptions, mainly in areas where the animal model database was limited. In clear agreement with animal models, Cd^{2+}, Zn^{2+}, and Cu^{2+} were demonstrated to be potent inducers of MT protein accumulation. In contrast to the similarity in MT protein expression, we obtained evidence that the human renal MT-2 gene has a unique pattern of regulation compared to both animal models and human-derived cell cultures. In the present study, we determined that MT-2A mRNA was not induced by exposure of HPT cells to Cd^{2+} or the other metals, a finding in contrast to studies in both animal models and other human cell culture systems in which a high level of MT-2 mRNA induction occurs upon exposure to Cd^{2+} or Zn^{2+}. While MT protein expression may be similar between humans and animal models, this finding provides initial evidence that regulation of the genes underlying MT protein expression may be divergent between species. *Key words*: cadmium, copper, gene regulation, lead, mercury, metallothionein, mRNA expression, nephrotoxicity, proximal tubule, silver, zinc. *Environ Health Perspect* 106:587–595 (1998). [Online 17 August 1998]

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The metallothioneins (MTs) are a family of low molecular weight, cysteine-rich intracellular proteins that bind transition metals, including Cu^{2+}, Zn^{2+}, Ag^{2+}, Hg^{2+}, and Cd^{2+} (*1*). MTs have been shown in numerous animal and human tissues and derived cell lines to be highly inducible by these metals and by a variety of other chemicals and stimuli (*2*). MTs have been proposed to play a role in metal detoxification (Cd^{2+}, Hg^{2+}, Ag^{2+}, and Pb^{2+}), in essential metal homeostasis (Zn^{2+} and Cu^{2+}), and in the protection of cells against damage induced by alkylating agents, oxygen radicals, and ionizing radiation (*3*). Of particular importance is the role MTs may play in mediating the adverse effects of the heavy metal pollutant Cd^{2+}. As summarized by Misra et al. (*4*), the particular target site sensitive to the toxic and carcinogenic effects of cadmium often depends on the species, strain, age, or sex of the animal used as a test subject. Furthermore, there is evidence that these differences in susceptibility to the adverse effects of Cd^{2+} may be related, at least in part, to the expression patterns of the MTs. Oberdorster et al. (*5*) demonstrated that expression of the MTs may mediate the species-specific susceptibility to Cd^{2+}-induced pulmonary carcinogenesis in rodents. Deficiencies in MT expression have

been implicated in susceptibility to Cd^{2+} carcinogenesis in mammalian testes (*6–8*) and the rat ventral prostate (*9,10*). While the basis for the majority of species- and strain-related differences in Cd^{2+} carcinogenicity and toxicity remains undefined in animals, the above studies provide evidence suggesting that deficiencies in MT gene expression may also underlie susceptibilities in the human.

Knowledge regarding the regulation of MT gene expression is even more limited in humans than in rodent models. This is partly due to the fact that the organization of the human MT gene family is more complex. In both mice and humans, there are four classes of MT genes, designated MT-1 through 4, based on sequence and charge characteristics. In the mouse, the genes encoding the MT-1 and MT-2 isoforms are both single copy genes and were the only known rodent MT genes prior to 1992. They are located approximately 6 kb apart on mouse chromosome 8, are coordinately regulated, and the proteins are thought to be functionally equivalent (*11,12*). Two additional single-gene members of the MT gene family have recently been identified and designated as MT-3 and MT-4; these are closely linked to, but

not coordinately regulated with, the other MT genes on mouse chromosome 8 (*13,14*). The mouse has no known MT pseudogene sequences as determined by Southern analysis. In contrast, the human MTs are encoded by a family of genes consisting of 10 functional (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3, MT-4) and 6 nonfunctional MT isoforms (MT-1C, MT-1D, MT-1I, MT-1J, MT-1K, MT-1L) located at 16q13, plus an additional processed pseudogene (MT-2B) located on chromosome 4 (*13–16*).

To address the possible alterations in MT gene regulation in humans, we began an analysis of the metal-induced expression of the MT gene family in cultures of human proximal tubule (HPT) cells. We chose this cell culture system because the kidney, and the proximal tubule in particular, represent an organ and cell type that are critically affected by chronic Cd^{2+} exposure in both animals and humans (*17–21*). In the present report, the expression of MT protein and MT-2A mRNA is determined as a function of metal exposure and cell toxicity in cultured HPT cells. The MT-2A gene was chosen for initial examination because it appears to be expressed ubiquitously and induced by metals in a wide variety of cells and tissues (*22*).

Materials and Methods

Cell culture. Stock cultures of HPT cells were grown in 75-cm^2 T-flasks using procedures recently detailed by this laboratory (*23*). The growth medium was a serum-free formulation, and the growth surface was treated with a collagen matrix to promote cell attachment and subculture. The cells were fed fresh growth medium every 3 days and were subcultured at confluence (normally 3–6 days post subculture) using trypsin (0.05%)-EDTA (0.02%). For use in experimental protocols, cells were subcultured at a 1:2 ratio, allowed to reach confluence (6 days following subculture), and

exposed to medium containing the various concentrations of metals. HepG2 and Hela cells were obtained from the American Type Culture Collection (Manassis, VA) and grown according to instructions.

Preliminary experiments were performed to determine the approximate concentrations of metal salts resulting in HPT cell toxicity over a 16-day period of exposure. Three concentrations of each metal were chosen so that over a 16-day time course at least one concentration would result in appreciable cell death. These concentrations were 9, 27, and 45 µM for Cd^{2+}; 20, 40, and 60 µM for Hg^{2+}; 50, 100, 150 µM for Ag^{2+}; 50, 100, and 200 µM for Pb^{2+}; 100, 200, and 300 µM for Zn^{2+}; and, 250, 500, and 750 µM for Cu^{2+}. Confluent HPT cells were treated with these metal concentrations and cell viability, MT protein, and MT-2A mRNA were determined at days 1, 4, 7, 10, 13, and 16 after metal exposure. In all instances the cells were fed every 3 days with media containing the appropriate concentration of metal. Three isolates of HPT cells were used to control for possible variations in metal susceptibility. These isolates were derived from normal cortical tissue obtained from kidneys removed for renal cell carcinoma. The kidneys were from a 72-year-old white female, a 63-year-old white male, and a 58-year-old white female. At least 35 primary cell cultures (75-cm^2 flasks) were obtained from each isolate and each had a culture lifespan of at least 17 passages at a 1:2 subculture ratio. All cultures displayed senescence by subculture 25. HPT cells between passages 5 and 8 were used in the present study.

All experiments were performed in triplicate. Statistical analysis was performed using SYSTAT software (Systat, Inc., Evanston, IL). Standard statistical tests included determinin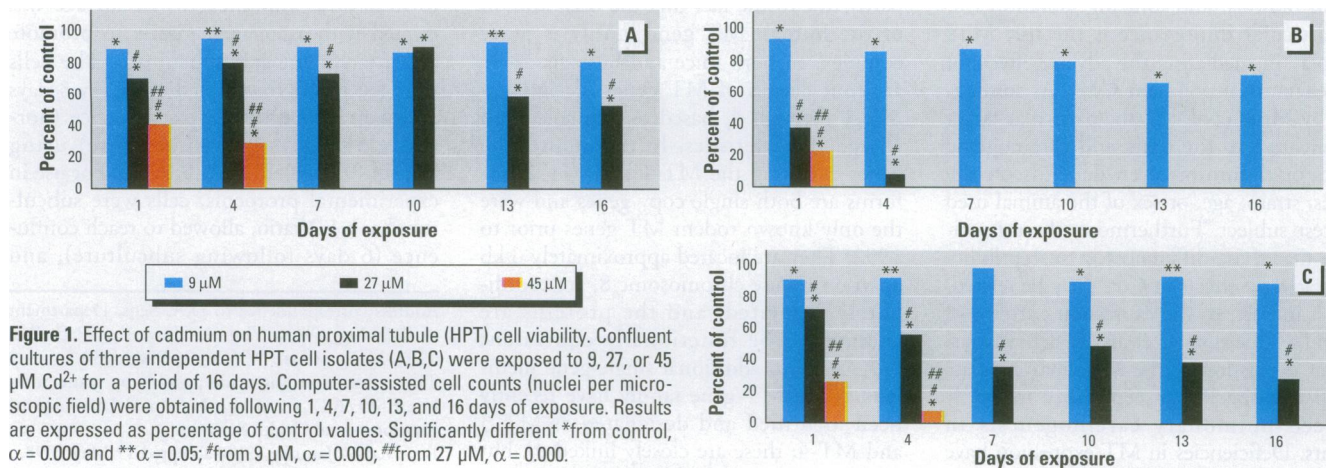g the mean and standard error of the mean. When multiple groups were compared, ANOVA was used to assess differences between groups using the Tukey HSD (honestly significance difference) test. Unless otherwise stated, differences were considered significant when $\alpha = 0.050$.

Cell viability. The effect of metal treatment on the viability of confluent cell monolayers was determined by the automated counting of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei of cells fixed and processed at days 1, 4, 7, 10, 13, and 16 of metal exposure. In this procedure, we used the nuclear stain DAPI and Kontron KS 400 image analysis software (Kontron Elektronik, Newport Beach, CA) as described previously (24). At the indicated time points, wells containing the monolayers were rinsed with phosphate-buffered saline (PBS), fixed for 15 min in 70% ethanol, rehydrated with PBS, and stained with 10 µl DAPI (10 µg/ml in distilled water). For analysis, each well was examined under epifluorescent illumination at 40× magnification on a Zeiss Axiovert 35 (Carl Zeiss Inc., Thornwood, NY) linked to the computer with an Optronics DEI 470 CCD camera (Optronics, Goleta, CA). For each time point, a minimum of 20 fields per well and three wells per data point were determined. Both nuclear counts and total nuclear area were obtained from the program and yielded equivalent results.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated according to the protocol supplied with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) as described previously (25). We determined the concentration and purity of the RNA samples using spectrophotometer scan in the UV region and ethidium bromide (EtBr) visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis. Total RNA (0.1 µg) was reverse transcribed using murine leukemia virus reverse transcriptase (50 units) in 1X PCR buffer (50 mM KCl

and 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$ solution, 20 units RNase inhibitor, 1 mM each of the dNTPs, and 2.5 mM random hexanucleotide primers. The samples were reverse transcribed for 20 min at 42°C, followed by a 5 min denaturation step at 99°C using a DNA thermocycler (Perkin-Elmer-Cetus 9600; Perkin-Elmer, Foster City, CA). The reverse transcribed product was then used for PCR amplification using the AmpliTaq DNA polymerase enzyme (2.5 units; Perkin-Elmer) and the specific upstream and downstream primers at a concentration of 0.075 µM each. The primers developed for analysis of each of the active MT genes have been previously described (25). The primers for analysis of MT-2A mRNA were upper 5′CCGACTCTAGC-CGCCTCTT3′ and lower 5′GTG-GAAGTCGCGTTCTTTACA3′, yielding a 259 bp product. Primers for the determination of glyceraldehyde 3-phosphate dehydrogenase (g3pdh) were obtained commercially (Clontech, Palo Alto, CA). The thermocycler was programmed to cycle at 95°C for a 2-min initial step, at 95°C for 15 sec, and at 68°C for 30 sec, with a final elongation step at 68°C for 7 min. Controls for each PCR included a no-template control in which water was added instead of the RNA and a no-reverse transcriptase control in which water was added instead of the enzyme. Samples were removed at 25, 30, 35, and 40 PCR cycles to ensure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers.

Restriction enzyme digestion of the MT-2A RT-PCR product. Following RT-PCR (50 µl reaction volume), 25 µl of PCR product was purified using the QiaQuik PCR Purification system (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol. DNA was eluted into 30 µl distilled H_2O. *Sgr*AI

Figure 1. Effect of cadmium on human proximal tubule (HPT) cell viability. Confluent cultures of three independent HPT cell isolates (A,B,C) were exposed to 9, 27, or 45 µM Cd^{2+} for a period of 16 days. Computer-assisted cell counts (nuclei per microscopic field) were obtained following 1, 4, 7, 10, 13, and 16 days of exposure. Results are expressed as percentage of control values. Significantly different *from control, $\alpha = 0.000$ and **$\alpha = 0.05$; #from 9 µM, $\alpha = 0.000$; ##from 27 µM, $\alpha = 0.000$.
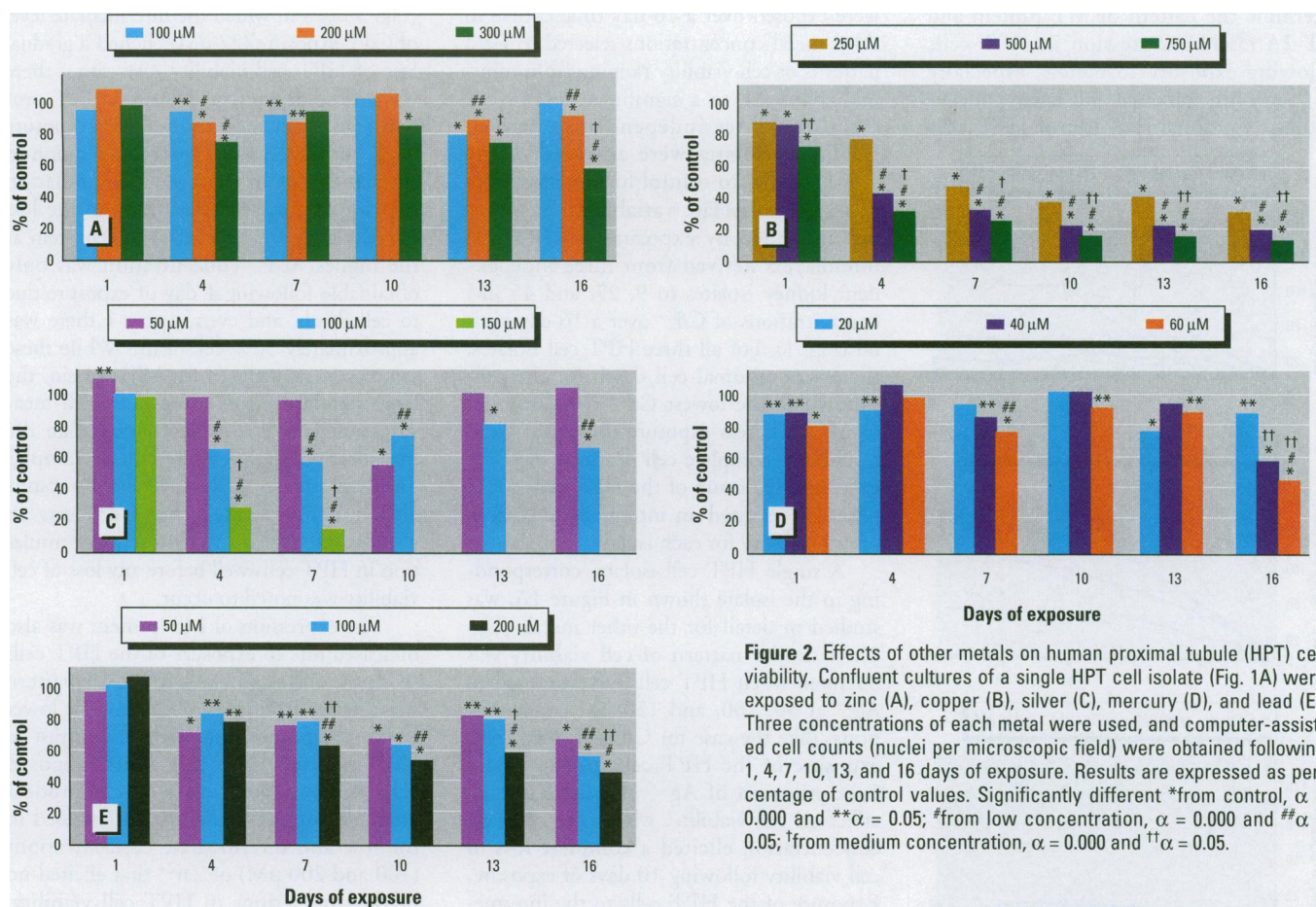
Figure 2. Effects of other metals on human proximal tubule (HPT) cell viability. Confluent cultures of a single HPT cell isolate (Fig. 1A) were exposed to zinc (A), copper (B), silver (C), mercury (D), and lead (E). Three concentrations of each metal were used, and computer-assisted cell counts (nuclei per microscopic field) were obtained following 1, 4, 7, 10, 13, and 16 days of exposure. Results are expressed as percentage of control values. Significantly different *from control, α = 0.000 and **α = 0.05; #from low concentration, α = 0.000 and ##α = 0.05; †from medium concentration, α = 0.000 and ††α = 0.05.

restriction digestion of MT- 2A was carried out by adding 15 μl DNA, 2 μl NE4 Buffer (New England Biolabs, Beverly, MA), distilled H_2O, and 20 units *sgr*AI enzyme (New England Biolabs) in a final reaction volume of 20 μl. Following incubation at 37°C for 1 hr, the reaction was terminated by adding 5 μl gel-loading buffer. Samples were electrophoresed on 2% agarose gels containing 0.5X TBE (0.045 M Tris-borate, 0.001 M EDTA) and 0.5 μg/ml EtBr.

MT protein determination. Cells were washed two times with PBS, harvested in buffer containing 10 mM Tris (pH 7.4) and 75 mM NaCl, and lysed by rapid freezing and thawing in liquid nitrogen. Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). For copper-induced samples, 1 mM dithiothreitol was added to the extract and total protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). MT protein was detected by immunoblotting using a mouse anti-horse antibody (DAKO-MT, E9; Dako, Carpinteria, CA) as the primary antibody following the procedure outlined by Mizzen et al. (*26*). Aliquots of 3 μg total protein were diluted to 75 μl in the above

harvest buffer and mixed with 75 μl of 3% glutaraldehyde to enhance the detection of MT. One hundred microliters of this mixture (2 μg total protein) was applied to a dot-blot apparatus with a polyvinylidine difluoride membrane. Samples were allowed to flow through the apparatus by gravity; this was followed by two PBS washes. After blocking with 10% skim milk in PBS, the membrane was soaked in primary antibody [1:100 dilution, 1% bovine serum albumin (BSA) in PBS] for 2 hr. The membrane was washed three times with PBS and anti-mouse alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI) was added (1:500 dilution, 0.4 % BSA in PBS). Antibody complexes were visualized using the alkaline phosphatase kit III (Vector, Burlingame, CA). Standard curves of rabbit liver Cd/Zn metallothionein-1 (Sigma, St. Louis, MO) were applied to each blot. MT protein was quantified by comparing the optical density of the dots to the standard MT curve using image analysis software (KS 400, Kontron). This assay has detection limits in the range of 0.1–0.5 ng MT protein.

Results

Effect of metal exposure on HPT cell viability. A goal of the present study was to
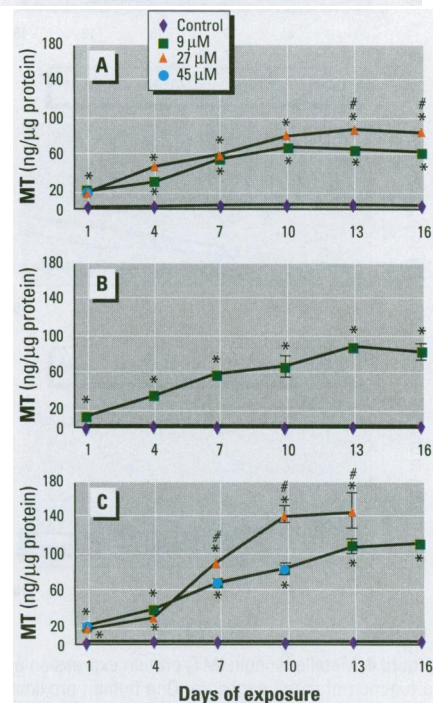

Figure 3. Metallothionein (MT) protein expression as a function of Cd exposure. Protein extracts were prepared from three independent HPT cell isolates (A,B,C) at the indicated time points and analyzed for MT protein by immunoblot. *Significantly different from control, α = 0.000; #significantly different from 9 μM concentration, α = 0.000.

determine the pattern of MT protein and MT-2A mRNA expression in HPT cells following exposure to metals, especially Cd^{2+}, at both sublethal and lethal concentrations. Confluent cultures of HPT cells
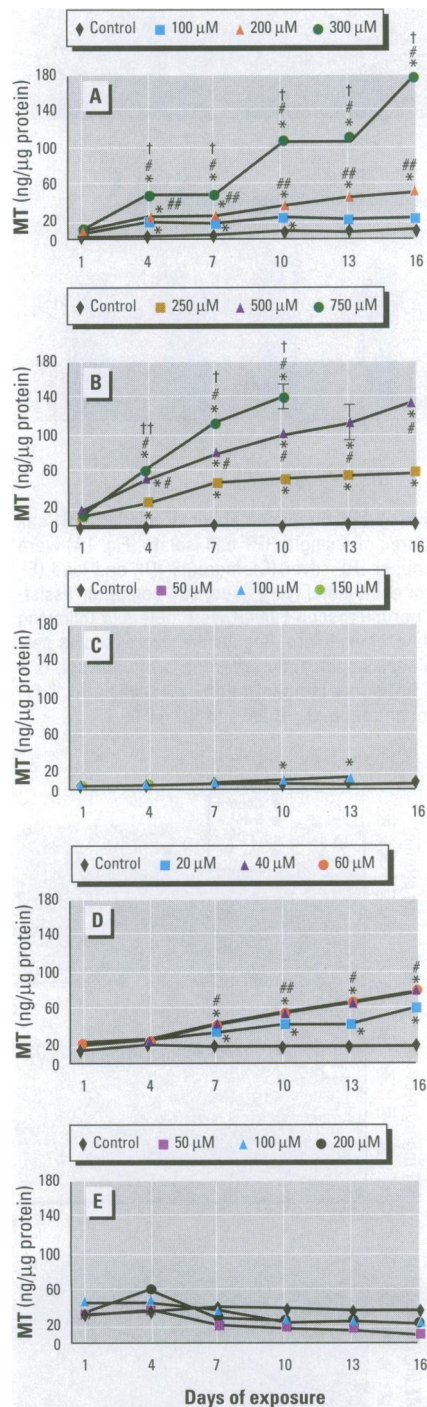

Figure 4. Metallothionein (MT) protein expression as a function of metal exposure. One human proximal tubule (HPT) cell isolate (Fig. 1A) was exposed to zinc (A), copper (B), silver (C), mercury (D), and lead (E) at the indicated exposure duration and metal concentrations. MT protein was determined by immunoblot. Significantly different *from control, α = 0.000 and ** α = 0.05; # from low concentration, α = 0.000 and ##α = 0.05; †from medium concentration, α = 0.000 and ††α = 0.05.

were exposed over a 16-day time course to three metal concentrations selected to yield patterns of cell viability ranging from minimal cell death to a significant loss of cell viability. Three independently derived HPT cell isolates were analyzed in the $CdCl_2$ studies to control for the possibility of isolate-to-isolate variability. This was accomplished by exposure of HPT cell monolayers derived from three independent kidney isolates to 9, 27, and 45 μM concentrations of Cd^{2+} over a 16-day period (Fig. 1). For all three HPT cell isolates, there was minimal cell death for the cells exposed to the lowest Cd^{2+} concentration (9 μM), whereas exposure to 45 μM Cd^{2+} resulted in complete cell death by day 7 of exposure. Exposure of the HPT cells to 27 μM Cd^{2+} elicited an intermediate pattern of cell viability for each isolate.

A single HPT cell isolate, corresponding to the isolate shown in Figure 1A, was studied in detail for the other metals (Fig. 2). A similar pattern of cell viability was obtained when HPT cells were exposed to Ag^{2+} at 50, 100, and 150 μM concentrations. Like the case for Cd^{2+}-exposed cells, exposure of the HPT cells to the lowest concentration of Ag^{2+} had little overall effect on cell viability, whereas the highest concentration elicited a complete loss of cell viability following 10 days of exposure. Exposure of the HPT cells to the intermediate concentration of Ag^{2+} elicited a moderate loss of cell viability. Exposure of HPT cells to the remaining four metal salts, Zn^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+}, also produced cell viability profiles with dose–response curves that included the range of minimal cell death to a significant loss of cell viability. At each time point in all the above cell viability studies, triplicate cultures were processed for the isolation of protein and total RNA.

Effect of metal exposure on the expression of MT protein. The level of MT protein expression was determined in extracts of HPT cells prepared at identical time points and with concentrations of metal exposures as defined above for the viability studies. The expression of MT protein was increased due to Cd^{2+} exposure in all three independent HPT cell isolates (Fig. 3). At the lowest concentration of Cd^{2+} (9 μM), where little loss of cell viability was noted, MT protein accumulation was similar among the three cell isolates. For each isolate, MT protein increased in a linear fashion over the initial 7–10 days of exposure, reaching a plateau by day 16 of exposure. Maximal accumulation of MT protein was also similar among the isolates at this level of Cd^{2+} exposure: 70, 90, and 110 ng/μg cell protein, respectively. In the isolates

(Fig. 3 A,C) in which the intermediate level of Cd^{2+} exposure (27 μM) elicited a gradual loss of HPT cell viability over time, there was a dose-dependent increase in MT protein levels compared to the lower exposure level, but there was no marked alteration in the pattern of MT protein accumulation that would correlate specifically to the loss of cell viability. The level of MT protein at the highest Cd^{2+} concentration was only obtainable following 1 day of exposure due to cell death, and even at day 1 there was approximately 50% cell death. While these samples were analyzed for MT protein, the large standard errors associated with measurement (the errors were larger than the measured values) rendered these samples unsuitable for analysis. Overall, the results clearly demonstrated that Cd^{2+} was an effective inducer of MT protein accumulation in HPT cells well before any loss of cell viability was noted to occur.

The expression of MT protein was also increased due to exposure of the HPT cells to Zn^{2+}, although the expression pattern was distinct from that of Cd^{2+} at the lower levels of exposure (Fig. 4A). In contrast to Cd^{2+}-exposed HPT cells, Zn^{2+}-exposed cells demonstrated only a very modest induction of MT protein when exposed to the low and intermediate concentrations (100 and 200 μM) of Zn^{2+} that elicited no marked reductions in HPT cell viability. However, at the highest concentration of Zn^{2+} exposure, where cell death was noted to occur late in the time course, the levels of MT protein were markedly induced, compared to both the lower concentrations and the control. Furthermore, the level of induction was comparable to that noted for HPT cells exposed to Cd^{2+}. The expression of MT protein was also increased due to exposure of the HPT cells to toxic concentrations of Cu^{2+} (Fig. 4B). The increase in MT protein accumulation was both dosage dependent and comparable in magnitude to that noted previously for the Cd^{2+}- and Zn^{2+}-exposed cells. Similar to Zn^{2+}-exposed HPT cells, MT protein accumulation was also blunted at the lowest level of Cu^{2+} exposure when compared to the increase obtained for Cd^{2+}-exposed cells.

In marked contrast to the large induction of MT protein accumulation found for HPT cells exposed to Cd^{2+}, Zn^{2+}, or Cu^{2+}, the expression of MT protein was only marginally increased, if at all, when the HPT cells were exposed to sublethal or lethal concentrations of Ag^{2+}, Hg^{2+}, or Pb^{2+} (Fig. 4C,D,E). For HPT cells exposed to Ag^{2+}, there was no increase in MT protein at the lowest level of exposure and only a very modest increase at the intermediate level of exposure, an exposure level where

loss of cell viability occurred late in the time course. The increase in MT protein in cells exposed to Ag^{2+} was less than 5% of that found for Cd^{2+}- exposed cells at similar time points and toxicities. For HPT cells exposed to Hg^{2+} (Fig. 4D), there was also only a very modest induction of MT protein compared to cells exposed to Cd^{2+}, Zn^{2+}, or Cu^{2+} at similar time points and toxicities. The very small increase in MT protein that did occur was not dosage dependent and the expression pattern demonstrated no obvious relationship to the loss of cell viability. For HPT cells exposed to Pb^{2+}, there was no increase in MT protein regardless of exposure level or loss of cell viability (Fig. 4E).

Determination of MT-2A mRNA. Accumulation of MT-2A mRNA was determined using an RT-PCR strategy based on the sequence diversity present in the 5' and 3' untranslated regions of the active MT genes (*25*). This strategy was chosen over Northern analysis due to the high degree of sequence homology among the active MT genes. This high degree of similarity among the nucleotide sequences of the 10 active MT genes makes it difficult to confirm if an individual probe for Northern analysis is specific for measuring a given individual MT isoform. The advantage of the RT-PCR strategy is that the resultant PCR product itself can be analyzed to determine if the primer pairs chosen for analysis were

indeed specific for the MT-2A mRNA. This was accomplished in the present study by determining the profile of restriction endonuclease cutting sites for all 10 active MT gene sequences and identifying a restriction site present only in the MT-2A sequence. For the MT-2A gene, a unique restriction site was identified as an *sgr*AI site (CR/CCGGYG) that would hypothetically cleave the 259 bp MT-2A RT-PCR product into two fragments (51 bp and 208 bp) if the reaction was specific for mRNA from the MT-2A gene. Using the noted MT-2A primers, this was tested on the RT-PCR products of the reaction initiated with 100 ng of total RNA obtained from normal human kidney tissue, from control HPT cells, and from HPT cells treated 24 hr with 45 µM Cd^{2+} (Fig. 5). It was demonstrated that each RNA source resulted in reaction products that were cleaved by the *sgr*AI restriction endonuclease into fragments of approximately 200 bp and a smaller fragment of approximately 50 bp, consistent with cleavage of the 259-bp MT-2A reaction product into 208- and 51-bp products as predicted by sequence information. In all cases, *sgr*AI digestion resulted in the complete disappearance of the 259-bp reaction product, indicating that the reaction product was composed entirely of the MT-2A sequence and not partially contaminated with a product generated by false priming. The negative control (MT-3 cDNA), which

has no *sgr*AI restriction site, was obtained from a previous study in which RT-PCR primers for MT-3 were shown to yield a legitimate MT-3 cDNA reaction product by a combination of both restriction endonuclease digestion by *Fok*I and direct sequencing (*27*).

Expression of MT-2A mRNA as a function of metal exposure. The level of MT-2A mRNA expression was determined on 0.1 µg
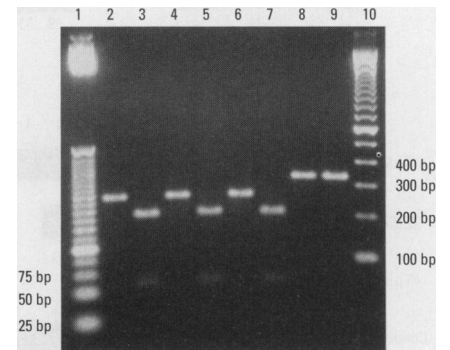

Figure 5. *Sgr*AI cleavage of MT-2A RT-PCR products from total RNA derived from human kidney tissue and cultured human proximal tubule (HPT) cells. Lanes 1 and 10 are base pair ladders; Lane 2, undigested product, kidney tissue; Lane 3, digested product, kidney tissue; Lane 4, undigested product, HPT cells; Lane 5, digested product, HPT cells; Lane 6, undigested product, Cd-treated (16 days at 27 µM Cd) HPT cells; Lane 7, digested product, Cd-treated HPT cells; Lane 8, undigested RT-PCR product for MT-3, HPT cells; Lane 9, digested RT-PCR product for MT-3, HPT cells.
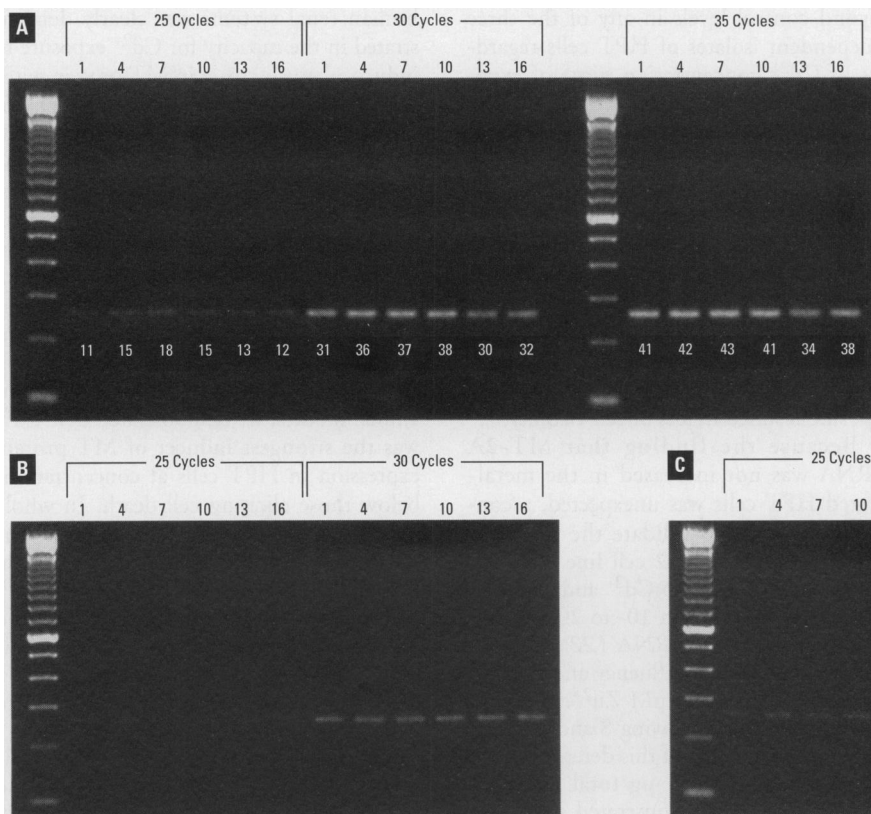

Figure 6. Evaluation of RT-PCR cycles for MT-2A mRNA. RT-PCR for MT-2A was performed on control RNA from three human proximal tubule (HPT) cell isolates (A,B,C) and sampled at various cycle numbers. Reaction products were electrophoresed on 2% agarose gels containing 0.5 µg/ml ethidium bromide. Integrated optical density (IOD) values [OD (inverted) × pixels²/1000] for individual bands are shown for A.
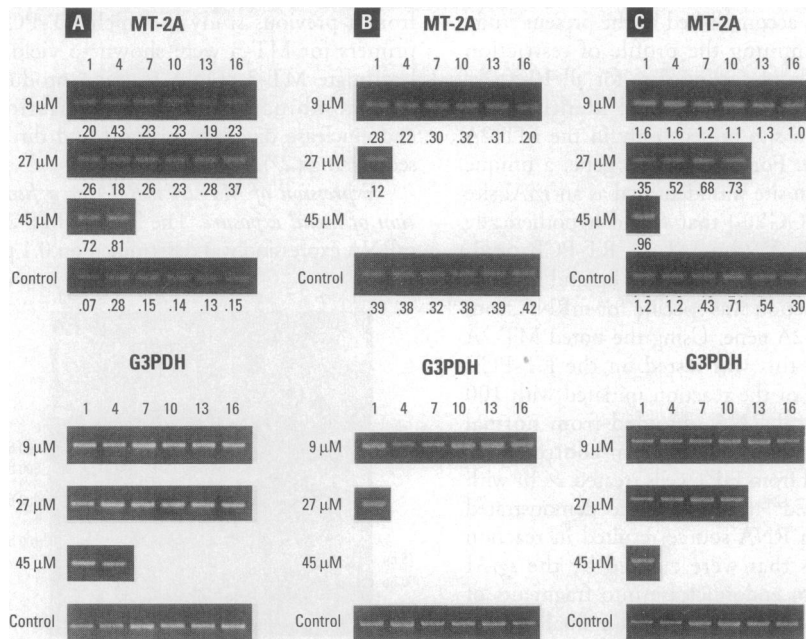
Figure 7. Expression of MT-2A mRNA in human proximal tubule (HPT) cells exposed to cadmium. RT-PCR for MT-2A and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was performed on three HPT cell isolates (A,B,C). Relative integrated optical density values [OD (inverted) × pixels²/1000] are shown for each isolate in the upper panel. The IOD value for each dose and time point was divided by the corresponding glyceraldehyde 3-phosphate dehydrogenase IOD value.

of total RNA isolated from HPT cells at identical time points and concentrations of metal exposures, as described above in the viability and MT protein expression studies. To ensure that MT-2A mRNA expression was determined in the linear region of the RT-PCR, reactions were stopped and samples removed for analysis at various time points. The need to analyze comparative RT-PCR in linear regions of the reaction cycle is illustrated effectively by the determination of MT-2A mRNA expression in control HPT cells unexposed to metals (Fig. 6). For the initial cell isolate used in this study, analysis of MT-2A mRNA was determined following 25, 30, and 35 PCR cycles primed with 0.1 µg of control total RNA samples obtained from HPT cells over the 16-day time course. Both visual analysis and integrated optical density measurements of the RT-PCR product intensities at 25, 30, and 35 reaction cycles clearly demonstrated that the PCR had reached saturation between cycles 30 and 35; indicating that results at these cycles would be unsuitable for comparative analysis (Fig. 6A). In contrast, the reaction product bands at cycle 30 indicate that the reaction is in the linear region and would be suitable for use in comparative RT-PCR. It was also confirmed for the other two HPT cell isolates that the PCR product at 30 cycles was in the linear range of the reaction (Fig. 6B,C).

The effect of Cd^{2+} exposure on the expression of MT-2A mRNA was also

determined at 30 reaction cycles using 0.1 µg of total RNA from the three independent isolates of HPT cells. A comparative analysis demonstrated that expression of MT-2A mRNA did not appear to increase beyond control levels in any of the three independent isolates of HPT cells regardless of Cd^{2+} concentration, time of exposure to Cd^{2+}, or whether there was a Cd^{2+}-induced loss of cell viability (Fig. 7). The failure of metal treatment to elicit a large increase in the accumulation of MT-2A mRNA was also confirmed for HPT cells exposed to Zn^{2+}, Cu^{2+}, Ag^{2+}, Hg^{2+}, and Pb^{2+} (Fig. 8). Similar to the case for Cd^{2+}-exposed cells, MT-2A mRNA levels were not increased over control by the other metals regardless of concentration, duration of exposure, or whether a given metal exposure resulted in loss of cell viability.

Because the finding that MT-2A mRNA was not increased in the metal-treated HPT cells was unexpected, a control was sought to validate the RT-PCR procedure. The HepG2 cell line was chosen because exposure to Cd^{2+} and Zn^{2+} has been shown to result in 10- to 20-fold elevations of MT-2A mRNA (22). HepG2 cells were grown to confluency and exposed to 9 µM Cd^{2+} or 100 µM Zn^{2+}, and total RNA was isolated following 8 and 24 hr of exposure. The results of this determination, using an input of 0.1 µg total RNA for RT-PCR, clearly demonstrated that both

Cd^{2+} and Zn^{2+} were effective inducers of MT-2A mRNA expression (Fig. 9). Control cells demonstrated a reaction product for MT-2A mRNA beginning at 26 reaction cycles. Both the Cd^{2+}- and Zn^{2+}-exposed cells demonstrated MT-2A reaction products beginning at 20 reaction cycles. These findings effectively demonstrate that the RT-PCR method used to determine MT-2A mRNA is valid and can detect an increase in MT-2A mRNA. A similar induction of MT-2A mRNA has also been reported for Hela cells (22). Hela cell MT-2A induction was also confirmed using the RT-PCR assay for MT-2A mRNA (data not shown).

Discussion

Many of the major features of renal MT protein expression expected from the accumulated knowledge gained from rodent animal models were demonstrated in cultures of HPT cells exposed to various metals. When differences were noted in the induction of MT protein expression between the cultured cells and the rodent animal models, these proved to be areas in which there was a limited database of rodent-based studies. In these instances, the results with cultured cells can be looked upon as extensions of the overall knowledge base of the renal effects of each given metal rather than defining large discrepancies between humans and animal models. A major shared feature of the rodent and human renal systems was clearly demonstrated in the capacity for Cd^{2+} exposure to induce a large increase in MT protein within the renal proximal tubule. In general, it has been shown in numerous rodent studies that Cd^{2+} is a potent inducer of the hepatic and renal MT-1 and MT-2 genes, which leads to large increases in MT protein accumulation (1,28–30). In the present study, exposure of HPT cells to both lethal and sublethal Cd^{2+} concentrations elicited greater than 20-fold accumulations of MT protein in a dose-dependent fashion over the time course. In accord with rodent animal models, we demonstrated that Cd^{2+} was the strongest inducer of MT protein expression in HPT cells at concentrations below those eliciting cell death. In whole animals, Cd^{2+} has been shown to be the strongest inducer of the metallothionein genes and protein, followed by Zn^{2+}, Ag^{2+}, Hg^{2+}, Bi^{2+}, and Cu^{2+}, with Ni^{2+}, Ca^{2+}, and Pb^{2+} being poor inducers (30). Thus, the pattern of MT protein expression for HPT cells exposed to Cd^{2+} was very similar to that expected from animal model studies.

The demonstration that HPT cells accumulated MT protein when exposed to Zn^{2+} is also in agreement with studies in
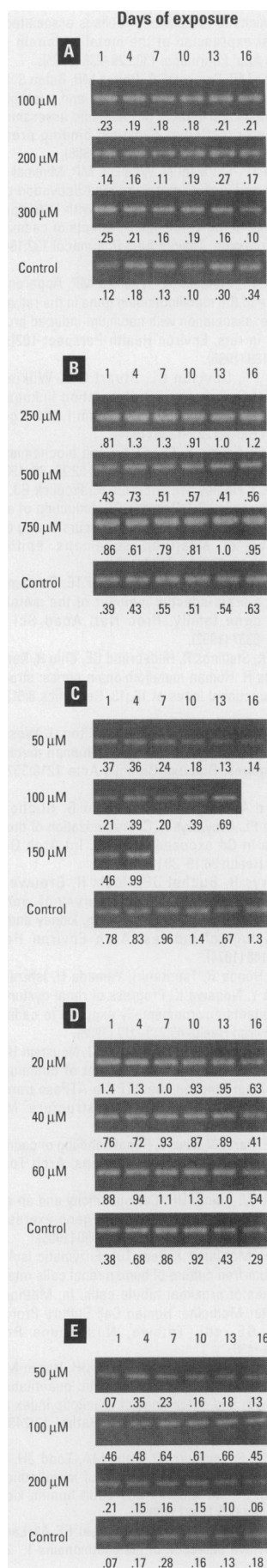
Figure 8. MT-2A mRNA expression following exposure to Zn (A), Cu (B), Ag (C), Hg (D), and Pb (E). RT-PCR for MT-2A was performed on total RNA from one HPT cell isolate (Fig. 1A) exposed to various metal concentrations over a 16-day time course, and reaction products are shown for days 1, 4, 7, 10, 13, and 16. Relative integrated optical density values [OD (inverted) × pixels²/1000] are shown for each metal exposure. The IOD value for each dose and time point was divided by the corresponding glyceraldehyde 3-phosphate dehydrogenase IOD value.

rodent models and derived cell cultures (1,2,28,29). The present studies with HPT cells also extended this observation. In HPT cells exposed to Zn^{2+}, we have shown that the degree of MT protein accumulation was influenced by the level of metal exposure. While the MT protein content of HPT cells was elevated significantly above control at Zn^{2+} concentrations eliciting little or no cell death, this level of induction was only modest when compared to the MT protein levels obtained when the cells were exposed to Cd^{2+} at nonlethal concentrations. In contrast, when HPT cells were exposed to Zn^{2+} at concentrations that elicited cell death late in the time course, the increase in MT accumulation was equal to or greater than that found for HPT cells exposed to Cd^{2+}. These findings are of interest in the human renal system because one of the functions ascribed to MT is to buffer intracellular Zn^{2+} concentrations and thereby attenuate toxicity and deficiency (31). Further evidence for this concept has come from recent studies with transgenic mice demonstrating that MT-1 and MT-2 protect against zinc deficiency and toxicity and that mice which overexpress MT are resistant to dietary zinc deficiency (32,33).

Although fewer studies document the ability of Cu^{2+} to induce MT in the rodent renal system, existing studies demonstrate the induction of MT protein accumulation by Cu^{2+}, although at levels 100% less effective than Zn^{2+} (34,35). As such, the finding that HPT cells accumulate MT protein as a result of Cu^{2+} exposure is consistent with current animal model studies. However, as noted for Zn^{2+}-exposed HPT cells, the current studies also demonstrated that the degree of MT protein accumulation by HPT cells exposed to Cu^{2+} was clearly influenced by metal exposure levels, either at lethal or sublethal concentrations. The accumulation of MT protein by Cu^{2+}-exposed cells was highest at concentrations that elicited cell death. Cu^{2+}-exposed HPT cells were also shown to accumulate similar quantities of MT protein as Cd^{2+}- and Zn^{2+}-exposed cells. The current findings in HPT cells suggest that the human proximal tubule is capable of a significant increase in

the accumulation of MT protein as Zn^{2+} or Cu^{2+} reach levels eliciting cell death.

There were also some differences between animal model data and the HPT cells that involved an inability of HPT cells to accumulate MT protein upon metal exposure. Most striking was the marginal induction of MT protein accumulation when HPT cells were exposed to Hg^{2+}. Several studies have shown that treatment of rats with either a single dose or repeated doses of inorganic Hg^{2+} causes the renal concentration of MT to increase (36–38). This increase occurs particularly in the outer cortex and outer stripe of the outer medulla and is presumably due to increased transcription of the MT genes (39). In contrast, an increase of this magnitude was not noted for HPT cells exposed to Hg^{2+} at either lethal or sublethal levels. A lack of induction of MT protein was also noted when the HPT cells were exposed to Ag^{2+} at both lethal and sublethal concentrations. Even though silver is classified as having minor toxicity, these findings are of interest because the intentional use of silver in medical and dental products increases potential exposure (40). However, the results regarding Ag^{2+} exposure are difficult to interpret, as there is only a very limited literature base detailing the response to Ag^{2+} exposure in general, and the MT response in particular, for both animals and humans (41,42). The present studies using HPT cells demonstrate that both Hg^{2+} and Ag^{2+} elicit only marginal, if any, MT protein accumulation.

The exposure of HPT cells to Pb^{2+} was designed to serve as a negative control for MT protein induction since exposure to
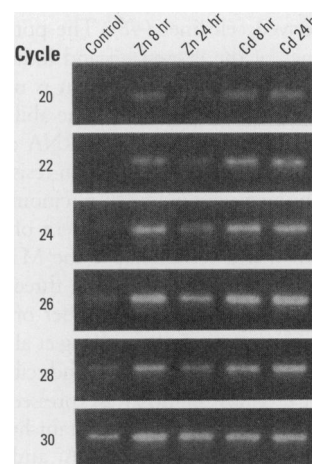

Figure 9. MT-2A mRNA expression in HepG2 cells following exposure to zinc or cadmium. RT-PCR for MT-2A was performed on RNA from cultured HepG2 cells exposed to zinc (100 µM) or cadmium (9 µM). RT-PCR products were sampled at 20, 22, 24, 26, 28, and 30 cycles. Products are shown for control cultures and cultures following 8 and 24 hr of metal exposure.

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Pb^{2+} has been reported to induce hepatic but not renal MT in animals (*34,43–47*). The findings with HPT cells were in agreement because no concentration of Pb^{2+}, lethal or sublethal, was noted to increase MT accumulation. If one ranks the ability of the metals to induce the accumulation of MT protein in HPT cells at exposure levels near lethal conditions, Cd^{2+}, Zn^{2+}, and Cu^{2+} are potent inducers with very similar efficiencies, while Hg^{2+}, Ag^{2+}, and Pb^{2+} demonstrate little, if any, significant induction of MT protein accumulation.

Even though the results regarding MT protein accumulation by HPT cells exposed to metals were similar to those noted in animal models, the regulation of the MT-2A gene in HPT cells appears unique compared both to animal models and to other human systems. In rodents, overwhelming evidence suggests that the hepatic and renal MT-1 and MT-2 genes are widely expressed and co-regulated by many agents including metals, hormones, and xenobiotics (*2,11*). We could find no instances in the literature of studies using rodent models or rodent tissue-derived cell cultures in which renal and hepatic MT-2 mRNA has not been demonstrated to be induced upon exposure to Zn^{2+} or Cd^{2+}. This also appears to be true in the human system, although for obvious reasons the induction of MT-2A mRNA via metals has been tested only under cell culture conditions. The initial observation of the induction of the human MT-2A gene by metals demonstrated that in 15 human-derived cell cultures, Cd^{2+} treatment resulted in a 10- to 30-fold induction of MT-2A mRNA (*22*). Induction of MT-2A mRNA by Cd^{2+} treatment has also been demonstrated for lymphoid-derived cell lines (*48*). The potential significance of the expression and induction of MT-2A mRNA in the human is underscored by the observation that the ability to induce expression of MT-2A mRNA correlates with development of cisplatin resistance in three human squamous cell carcinoma cell lines (*49*). In this study, the pattern of gene expression was determined for the MT-2A, MT-1A, B, E, F, and G genes in three pairs of cell lines in which one member of each pair was resistant to cisplatin. Yang et al. (*49*) found that MT-2A mRNA was inducible by zinc, that the most highly overexpressed MT isoform in all three cisplatin resistant human carcinoma cell pairs was MT-2A, and that no other isoform was universally overexpressed. This finding was interpreted as being consistent with a role for the MT-2A isoform in mediating cisplatin resistance. To our knowledge, the finding that MT-2A mRNA accumulation was not increased by Cd^{2+} exposure in HPT cells is the first demonstration that the human MT-2A gene

can be refractory to heavy metal treatment. Proof that RT-PCR was specific for MT-2A mRNA and that increased accumulation could be detected was provided by two procedures. First, an advantage provided by RT-PCR is the ability to recover the reaction product from the agarose gel and to subject this product to further analysis to confirm identity of a given gene. In the present study, this advantage was used to determine that the MT-2A RT-PCR product contained a *sgr*AI restriction endonuclease cleavage site, a site known from sequencing data to be present only in the MT-2A gene of the human MT family. The demonstration that *sgr*AI digestion completely cleaved the MT-2A reaction product into appropriate fragments provides a high degree of confidence that the primers developed for MT-2A mRNA and the RT-PCR were specific for the transcription product of the MT-2A gene. Proof that the RT-PCR protocol could detect an increased accumulation of MT-2A mRNA relied on previous studies which demonstrated that the HepG2 cell line, when exposed to either Cd^{2+} or Zn^{2+}, displayed a 10- to 20-fold accumulation of MT-2A mRNA (*22*). Utilizing the HepG2 cell line and exposures to Cd^{2+} and Zn^{2+}, the RT-PCR protocol that we used demonstrated a large increase in the amount of accumulated MT-2A mRNA for treated versus control cells.

The present study demonstrates strong similarities between the human and rodent renal system in the ability to accumulate MT protein as a result of metal exposure. However, the studies also provide initial evidence that this accumulation of MT protein may be mediated through distinctly different patterns of gene regulation between humans and animals.

REFERENCES AND NOTES

1. Hamer DH. Metallothionein. Annu Rev Biochem 55:913–951 (1986).
2. Kägi JHR. Evolution, structure and chemical activity of class I metallothioneins: an overview. In: Metallothionein III. Biological Roles and Medical Implications (Suzuki KT, Imura N, Kimura M, eds). Berlin:Birkhauser Verlag, 1993;29–56.
3. Cherian MG, Howell SB, Imura N, Klaassen CD, Koropatnick J, Lazo JS, Waalkes MP. Role of metallothionein in carcinogenesis. Toxicol Appl Pharmacol 126:1–5 (1994).
4. Misra RR, Crance KA, Bare RM, Waalkes MP. Lack of correlation between the inducibility of metallothionein mRNA and metallothionein protein in cadmium-exposed rodents. Toxicology 117:99–109 (1997).
5. Oberdorster G, Cherian MG, Baggs RB. Correlation between cadmium-induced pulmonary carcinogenicity, metallothionein expression, and inflammatory processes: a species comparison. Environ Health Perspect 102(suppl 3):257–263 (1994).
6. Melo IM, Bare RM Waalkes MP. Metallothionein gene expression in testicular interstitial cells and liver of rats treated with cadmium. Toxicology 107:121–130 (1996).
7. Shiraishi N, Hochadel JF, Coogan TP, Koropatnick J, Waalkes MP. Sensitivity to cadmium-induced genotoxicity in rat testicular cells is associated with minimal expression of the metallothionein gene. Toxicol Appl Pharmacol 130:229–236 (1995).
8. Waalkes MP, Perantoni A, Bhave MR, Rehm S. Strain dependence in mice of resistance and susceptibility to the testicular effects of cadmium: assessment of the role of testicular cadmium binding proteins. Toxicol Appl Pharmacol 93:47–61 (1988).
9. Coogan TP, Shiraishi N, Waalkes MP. Minimal basal activity and lack of metal-induced activation of the metallothionein gene correlates with lobe-specific sensitivity to the carcinogenic effects of cadmium in the rat prostate. Toxicol Appl Pharmacol 132:164–173 (1995).
10. Coogan TP, Shiraishi N, Waalkes MP. Apparent quiescence of the metallothionein gene in the rat ventral prostate: association with cadmium-induced prostate tumors in rats. Environ Health Perspect 102(suppl 3):137–139 (1995).
11. Searle PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD. Regulation linkage and sequence of mouse metallothionein I and II genes. Mol Cell Biol 4:1221–1230 (1984).
12. Kägi JHR, Kojima Y. Chemistry and biochemistry of metallothionein. Experimenta Suppl 52:25–80 (1987).
13. Quaife CJ, Findley SD, Erickson JC, Froelick GJ, Kelly EJ, Zambrowicz BP, Palmiter RD. Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. Biochemistry 33:7250–7259 (1994).
14. Palmiter RD, Findley SD, Whitmore TE, Durnam DM. MT-III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci USA 89:6333–6337 (1992).
15. West AK, Stallings R, Hildebrand CE, Chiu R, Karin M, Richards R. Human metallothionein genes: structure of the functional locus at 16q13. Genomics 8:513–518 (1990).
16. Stennard FA, Holloway AF, Hamilton J, West AK. Characterization of six additional human metallothionein genes. Biochim Biophys Acta 1218:357–365 (1994).
17. Bernard A, Roels H, Hubermont G, Buchet JP, Masson PL, Lauwerys R. Characterization of the proteinuria in Cd exposed workers. Int Arch Occup Environ Health 38:19–30 (1976).
18. Lauwerys R, Buchet JP, Roels H, Brouwers J, Stanescu D. Epidemiological survey of workers exposed to cadmium: effect of lung, kidney and several biological indices. Arch Environ Health 28:145–148 (1974).
19. Kido T, Honda R, Tsuritani I, Yamada H, Ishizaki M, Yamada Y, Nogawa K. Progress of renal dysfunction in inhabitants environmentally exposed to cadmium. Arch Environ Health 43:213–217 (1988).
20. Gonich HC, Indraprasit S, Rosen VJ, Neustein H, Van de Velde R, Raghavan SRV. Effect of cadmium on renal tubular function, the ATP-Na-ATPase transport system and renal tubular ultrastructure. Miner Electrolyte Metab 3:21–35 (1980).
21. Bosco E, Porta N, Diezi J. Renal handling of cadmium: a study by tubular microinjections. Arch Toxicol 7:371–373 (1986).
22. Schmidt CJ, Hamer DH. Cell specificity and an effect of *ras* on human metallothionein gene expression. Proc Natl Acad Sci USA 83:3346–3350 (1986).
23. Todd JH, McMartin K Sens DA. Enzymatic isolation and serum-free culture of human renal cells retaining properties of proximal tubule cells. In: Methods in Molecular Medicine: Human Cell Culture Protocols (Jones GE, ed). Totawa, NJ:Humana Press, 1996;431–436.
24. Tarnowski BI, Sens DA, Nicholson JH, Hazen-Martin DJ, Garvin AJ, Sens MA. Automatic quantitation of cell growth and determination of mitotic index using DAPI nuclear staining. Pediatr Pathol 13:249–265 (1993).
25. Mididoddi S, McGuirt JP, Sens MA, Todd JH, Sens DA. Isoform-specific expression of metallothionein mRNA in the developing and adult human kidney. Toxicol Lett 685:17–27 (1996).
26. Mizzen CA, Cartel NJ, Yu WH, Fraser PE, McLachlan DR. Sensitive detection of metallothioneins-1, -2 and

-3 in tissue homogenates by immunoblotting: a method for enhanced membrane transfer and retention. J Biochem Biophys Methods 32:77–83 (1996).

27. Hoey JG, Garrett SH, Sens MA, Todd JH, Sens DA. Expression of MT-3 mRNA in human kidney, proximal tubule cell cultures, and renal cell carcinoma. Toxicol Lett 92:149–160 (1997).

28. Kägi JHR, Schäffer A. Biochemistry of the metallothioneins. Biochemistry 27:8509–8515 (1988).

29. Koropatnick J, Leibbrandt MEI. Effects of metals on gene expression. In: Toxicology of Metals (Goyer RA, Cherian MG, eds). Berlin:Springer, 1995;93–120.

30. Beyersmann D, Hechtenberg S. Cadmium, gene regulation, and cellular signaling in mammalian cells. Toxicol Appl Pharmacol 144:247–261 (1997).

31. Bremmer I. Nutritional and physiologic significance of metallothionein. Methods Enzymol 205:25–35 (1991).

32. Dalton T, Fu K, Palmiter RD, Andrews GK. Transgenic mice that overexpress metallothionein-I resist dietary zinc deficiency. J Nutr 126:825–833 (1996).

33. Kelly EJ, Quaife CJ, Froelick J, Palmiter RD. Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. J Nutr 126:1782–1790 (1996).

34. Waalkes MP, Klaassen CD. Concentration of metallothionein in major organs of rats after administration of various metals. Fundam Appl Toxicol 5:473–477 (1985).

35. Irato P, Sturniolo GC, Giacon G, Magro A, D'Inca R, Mestriner C, Albergon V. Effect of zinc supplementation on metallothionein, copper, and zinc concentration in various tissues of copper-loaded rats. Biol Trace Elem Res 51:87–96 (1996).

36. Piotrowski JK, Trojanowska B, Wisniewska-Knypl JM, Bolanowska W. Mercury binding in the kidney and liver or rats repeatedly exposed to mercuric chloride. Toxicol Appl Pharmacol 27:11–19 (1974).

37. Zalups RK, Cherian MG. Renal metallothionein metabolism after a reduction of renal mass. I. Effect of unilateral nephrectomy and compensatory renal growth on basal and metal-induced renal metallothionein metabolism. Toxicology 71:83–102 (1992).

38. Morcillo MA, Santamaria J. Mercury distribution and renal metallothionein induction after subchronic oral exposure in rats. Biometals 9:213–220 (1996).

39. Zalups RK, Lash LH. Advances in understanding the renal transport and toxicity of mercury. J Toxicol Environ Health 42:1–44 (1994).

40. Hollinger MA. Toxicological aspects of topical silver pharmaceuticals. Crit Rev Toxicol 26:255–260 (1996).

41. Shinogi M, Maeizumi S. Effect of preinduction of metallothionein on tissue distribution of silver and hepatic lipid peroxidation. Biol Pharm Bull 16:372–377 (1993).

42. Liu J, Kershaw WC, Klaassen CD. The protective effect of metallothionein on the toxicity of various metals in rat primary hepatocyte culture. Toxicol Appl Pharmacol 107:27–34 (1991).

43. Durnam DM, Palmiter RD. Induction of metallothionein-1 mRNA in cultured cells by heavy metals and iodoacetate: evidence for gratuitous inducers. Mol Cell Biol 4:484–491 (1984).

44. Goering PI, Fowler BA. Metal constitution of metallothionein influences inhibition of delta aminolaevulinic acid dehydratase porphobilinogen synthetase by lead. Biochem J 245:339–345 (1987).

45. Maitani T, Watahiki A, Suzuki KT. Induction of metallothionein after lead administration by three injection routes in mice. Toxicol Appl Pharmacol 83:211–217 (1986).

46. Ikebuchi H, Teshima R, Suzuki K, Sawada JI, Terao T, Yamane Y. An immunological study of a lead-thionein-like protein in rat liver. Biochem Biophys Res Comm 136:535–541 (1986).

47. Ikebuchi H, Teshima R, Suzuki K, Terao T, Yamane Y. Simultaneous induction of lead-metallothionein-like protein and zinc-thionein in the liver of rats given lead acetate. Biochem J 233:541–546 (1986).

48. Pellegrini O, Davenas E, Morin L, Tsangaris GT, Benveniste J, Manuel Y, Thomas Y. Modulation of stress proteins by Cd^{2+} in human T cell line. Eur J Pharmacol 270:221–228 (1994).

49. Yang Y-Y, Woo ES, Reese CE, Bahnson RR, Saijo N, Lazo JS. Human metallothionein isoform gene expression in cisplatin-sensitive and resistant cells. Mol Pharmacol 45:453–460 (1994).

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