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Basal and Zinc-Induced Metallothionein in Resistance to Cadmium, Cisplatin, Zinc, and *tert*Butyl Hydroperoxide: Studies Using MT Knockout and Antisense-Downregulated MT in Mammalian Cells

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Metallothioneins (MTs) mediate resistance to metal and non-metal toxicants. To differentiate the role of MTs from other protective factors, resistance to zinc (Zn), cadmium (Cd), *tert*butyl hydroperoxide (*t*BH), and cisplatin (CDDP) was compared in renal cell lines from wild type (MT-WT) and MT-1/MT-2 knockout (MT-KO) mice. MT-WT cells were more resistant to *t*BH than MT-KO cells but, unexpectedly, were more sensitive to Zn, Cd, and CDDP. Thus, basal expression of MT conferred resistance to *t*BH, but not to Cd or CDDP. Pretreatment with Zn increased MT expression and enhanced resistance to Cd and CDDP only in MT-WT cells, indicating a critical role for MT in this form of resistance. By contrast, Zn-pretreatment increased resistance to subsequent Zn exposure, but did not alter resistance to *t*BH, regardless of MT-status. Therefore, Zn-induced resistance to subsequent exposure to Zn (but not to Cd or CDDP) was mediated by non-MT factors, and neither Zn-induced MT nor other factors affected *t*BH sensitivity. Furthermore, antisense down-regulation of MT in human HeLa cells reduced basal MT levels and resistance to TBH, but not to Cd or CDDP. Therefore, basal MT alone can mediate resistance to TBH (but not to Cd or CDDP) in mouse and human cells. These data suggest that MT can mediate resistance to toxicants by different mechanisms, some of which correlate with the cellular content of MT protein. Moreover, resistance to some agents (Cd and CDDP) can be enhanced by inducing MT. Resistance to other agents (*t*BH) requires only basal (non-induced) MT levels.

Key Words: **metallothionein; knockout; antisense; drug sensitivity; drug resistance; cisplatin; cadmium; reactive oxygen species; tertbutyl hydroperoxide; zinc.**

Metallothioneins (MTs) are small (less than 10 kDa), cysteine-rich, metal-inducible, and metal-binding proteins that protect against metal toxicity. MTs have also been suggested to protect against toxic and carcinogenic events mediated by a broad range of non-metal toxicants (Sato and Bremner, 1993; Theocharis *et al.*, 2003). Nuclear MT has been proposed to regulate and/or protect against nuclear oxidant events during the cell cycle: MTs can bind and inactivate reactive oxygen intermediates (ROI) and can protect against ROI damage (Takahashi *et al.*, 2005; Tamai *et al.*, 1993). MTs may also participate in physiological events other than resistance to toxicants: MT levels vary during development (including changes in intracellular compartmentalization), proliferation, cellular hypertrophy, and macrophage activation (Haq *et al.*, 2003). While it is likely that MTs protect against metal- and ROI-induced toxicity by sequestering toxic species (Theocharis *et al.*, 2003), a role in mediating the activity of Zn-requiring transcription factors that regulate cellular susceptibility to damage and apoptosis has also been proposed (Feng *et al.*, 2005; Haq *et al.*, 2003). Therefore, the mechanism by which MTs mediate protective effects and resistance to toxic agents is likely complex and variable among cell types and toxicants.

MT expression has been correlated with resistance to Cd and other toxic metal ions and chemicals in many studies where MTs are overexpressed by virtue of induction with metals, cytokines, hormones, and other chemicals (Blain *et al.*, 1998; Schroeder and Cousins, 1990). Induced MT protein is believed to be the key mediator of induced cellular protection. However, experiments where MT expression was increased in the absence of induction (after transfection of mammalian MT expression vectors) suggest that MT is not always the critical induced resistance factor. For example, increased MT in transgenic mice, or Zn supplementation of both wild type and MT-knockout animals, did not reduce the hepatotoxic effects of carbon tetrachloride (Davis *et al.*, 2001). Thus, MT overexpression may not provide additional protection beyond that afforded by low, basal MT levels.

Intracellular localization of MT has been reported to be an important determinant of resistance to Cd and agents that

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induce oxidative stress (Levadoux-Martin *et al.*, 2001). Furthermore, enhanced MT expression may have a negative effect on cell growth in the absence of exposure to toxicants, and may decrease, rather than increase, cellular resistance to toxic agents such as Cd (Koropatnick and Pearson, 1993; Robson *et al.*, 1992). A number of studies have utilized mice, in which MT expression was ablated or enhanced (MT knockout and transgenic strains, respectively). The findings from of these studies support a role for MT in protection against multiple stresses (Kang, 1999; Liu *et al.*, 1998; Rofe *et al.*, 1998). By comparison, others have suggested that MT does not protect (DiSilvestro *et al.*, 1996; Itoh *et al.*, 1997; Kaina *et al.*, 1990; Liu *et al.*, 1999). Overall, the role of MT in mediating resistance to toxicants appears to differ depending on whether MT function is assessed in the context of low, un-induced, basal MT; MT expression mediated by transfected expression vectors in the absence of induction; or MT induced by various agents that stimulate transcription of endogenous MT genes and other events, unrelated to MT, affected by induction.

In the present study, we tested the hypothesis that toxicant resistance in cells with low basal MT (compared to cells without MT or with less-than-basal expression, generated by gene knockout or antisense treatment, respectively), depends on both MT and non-MT factors. We also tested the hypothesis that MT protects against multiple toxicant-specific mechanisms requiring the presence of MT, but may be independent of the level of MT, and that Zn pretreatment triggers multiple cellular events (in addition to MT induction) that may also mediate resistance. To test these hypotheses, we compared the sensitivity to Cd and Zn (non-essential and essential metals, respectively), CDDP (a chemotherapeutic drug for which resistance is associated with MT overexpression) (Rosell and Felip, 2001), and *tert*butyl hydroperoxide (*t*BH, a generator of toxic reactive oxygen) in mouse MT-null and MT-WT cells. To resolve the role of MT among other Zn-induced events, MT-null and MT-WT cells were studied before and after pretreatment with Zn. To extend our investigation to human cells, we downregulated MT using antisense RNA expression in a human cell line and assessed the effect on resistance to multiple toxic stresses. Our findings indicate that MT mediates resistance to different agents by various mechanisms, some of which correlate with MT content and are enhanced by MT induction, while others require only low, un-induced MT levels, which are not elevated by Zn pretreatment.

MATERIALS AND METHODS

Chemicals and Reagents

$ZnCl_2$ was obtained from BDH Laboratory Supplies (Toronto, ON); $CdCl_2$ and *tert*-butyl hydroperoxide (*t*BH) were purchased from Sigma Chemical Company (St. Louis, MO). CDDP was obtained from Bristol Laboratories of Canada (Montreal, Quebec).

Cell Culture

MT-WT, MT-KO1, and MT-KO2 cell lines were generated from embryonic kidneys from control mice (129/Sv strain), and MT-null mice with disrupted production of MT-1 and MT-2 proteins (129/Sv-Mt1Mt2^{tm1Br} strain), respectively (Masters *et al.*, 1994) as described previously (Butcher *et al.*, 2004). Both MT-KO1 and MT-KO2 cell lines express MT-1 and MT-2 mRNA but lack MT-1 and MT-2 protein (both basal and inducible with Zn or Cd). All three cell lines have a phenotype typical of fibroblasts and both MT-KO1 and MT-KO2 cells do not have altered expression of MT-3 and MT-4 mRNAs compared with that in the MT-WT cells (Butcher *et al.*, 2004). All cell lines were maintained in DMEM/10% FBS without antibiotics at 37° in 5% CO_2.

HeLa cells express MT-1 and MT-2 and are a highly transfectable human cell line amenable to antisense downregulation (Berg *et al.*, 2002). HeLa cells, grown in culture conditions identical to those used for mouse MT-WT and MT-KO cell lines, were transfected with pNMH-asMT. This is a *neo*-expressing, eukaryotic expression vector employing a metallothionein promoter/enhancer to drive expression of the entire 400 bp murine MT-1 cDNA inserted, in reverse orientation, into the BamH1 multiple cloning site) or an empty control pNMH vector (Khokha and Denhardt, 1987; the gift of Dr. Rama Khokha, Univ. Health Network, Toronto, Canada). We have reported previously that this cDNA, when expressed in reverse orientation in a different vector, has the capacity to reduce MT mRNA and protein levels (Leibbrandt *et al.*, 1994). After 24 h the transfected cells were treated with G418 (Gibco BRL, Grand Island, NY) to select for cells containing vector. Colonies that formed in the presence of G418 were selected and analyzed for the presence of pNMH-asMT. Five control (vector alone) cell lines and three cell lines containing antisense MT-1 cDNA were identified and isolated.

All cell lines were maintained in DMEM supplemented with 10% FBS in a 37°C incubator under 5% CO_2.

RNA Extraction and Analysis

Cells were seeded in 90 mm dishes (2×10^6 cells) and allowed to grow for 48 h. The cells were collected by addition of TRIzol Reagent (Gibco BRL, Grand Island, NY) followed by scraping with a rubber policeman. RNA was isolated as described by the manufacturer. Isolated RNA was dissolved in RNAse-free water and stored at −80°C until use.

Northern blots

Heme oxygenase-1 (HO-1), γ-glutamyl-cysteine synthetase (γ-GCS), Cu/Zn superoxide dismutase (SOD-1) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) transcripts were detected by Northern blot analysis. The HO-1 cDNA probe was a 953 bp murine HO-1 fragment within a 1.5 kb insert of plasmid pT7T3-Pac-HO1 (Marra *et al.*, 1996a; obtained from the American Type Culture Collection, Manassas, VA). The γ-GCS cDNA probe, complementary to both mouse and rat γ-GCS, was a 390 bp insert isolated after EcoR1 digestion of pGCS (Borroz *et al.*, 1994; the gift of T. Kavanagh, Univ. of Washington, Seattle, WA). The SOD-1 cDNA probe was a 430 bp sequence within a 900 bp fragment isolated after Xho1 cleavage of plasmid pME18S-FL3-SOD1 (American Type Culture Collection, Manassas, VA). The GAPDH cDNA probe was a 1050 bp insert of plasmid pM-GAP (the gift of A. Chambers, London Regional Cancer Program, London, Ont., Canada). Probe hybridization to electrophoresis bands were visualized using a PhosphorImager SI (Molecular Dynamics, Inc., Sunnyvale, CA) and band intensity was determined using the PhosphorImager data file ImagQuant Analysis software (Molecular Dynamics, Inc., Sunnyvale, CA).

Reverse transcription/polymerase chain reaction (RT-PCR)

RT-PCR was used to detect the levels of murine antisense MT-1 being expressed in the stably-transfected HeLa cells. Briefly, three aliquots (1 μg each) of RNA from each analyzed RNA sample was lyophilized and redissolved in 20 μl of reverse transcription mix (10 μl RNAse-free water, 1 μl random hexanucleotide primers [100 pmoles/μl], 4 μl 5X reverse transcription buffer, 2 μl of 10 mM mixed deoxyribonucleotides [dNTPs],

1 µl of Moloney murine leukaemia virus [MMLV] reverse transcriptase and 2 µl of 0.1 M dithiothreitol [DTT]). The reaction components were supplied by Gibco BRL Life Technologies (Grand Island, NY). Each tube was incubated at 37°C for 60 min followed by 5 min at 95°C. Five µl of the resulting cDNA products were amplified by PCR (94°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 30 s) in 45 µl PCR mix (5 µl Taq DNA polymerase buffer, 1.5 µl of 50 mM MgCl2, 1 µl of 10 mM mixed dNTPs, 0.5 µl of each primer (50 pmoles/µl), 0.25 µl of Taq polymerase and 36.25 µl of sterile water to make a final volume of 50 µl. The oligonucleotide primers were synthesized at the MRC Molecular Biology Core Facility, London Regional Cancer Centre. Primers were generated to specifically amplify mouse antisense MT-1 RNA transcribed from the pNMH/asMT-1 vector in human HeLa cells. The primer sequences were as follows:

Forward: 5′-CGGATCCAGACTCAAACAGGCTTTTAT-3′
Reverse: 5′-CGGATCCCGGAATGGACCCCAACTGCT-3′

MT Protein Measurement

Cells were lysed in 200 ul of 1% Tween 20 (BDH, Toronto, ON) in PBS by five cycles of freezing (isopropanol/dry ice) and thawing (65°C). Insoluble protein was removed by centrifugation. A sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) was used to quantify MT protein concentrations as previously described (Butcher *et al.*, 2004). Standard rabbit MT-2 (Sigma Chemical Co., St Louis, MO), mouse monoclonal antibody to MT (Clone E9; IgG1, kappa isotype)(DAKO Corp., Carpenteria, CA), europium-labelled anti-mouse IgG antibody and DELFIA assay buffer and Enhancement solution (Wallac Oy, Turku, Finland), were used according to the manufacturer's protocol. The amount of MT protein was expressed relative to total soluble protein (estimated by a modification of the Bradford assay; Bradford, 1976) as described by the manufacturer (Bio-RDA protein assay, Bio-RDA Laboratories, Hercules, CA).

Proliferation Assay

MT-WT, MT-KO1, and MT-KO2 cells were plated at 1×10^4 cells/well in 24-well tissue culture plates. Proliferation was assessed on days 1 through 4 at which times the media was removed and the cells were washed twice with PBS. The cells were trypsinized and the number of cells determined with a Coulter Counter Z_1. Doubling times were determined, in triplicate experiments, from the slope of logarithmic plots of cell number versus time during exponential growth phase (48 to 72 h after plating).

Measurement of Toxicity

Cytotoxicity of $ZnCl_2$ (0–200 µM), $CdCl_2$ (0–2 µM), CDDP (0–2 µM), and *t*BH (0–25 µM) was assessed by capacity to inhibit growth of cells in complete medium, as described below.

Growth Inhibition Assay

Basal resistance to toxicity. Cells (2×10^4 cells/well) were seeded in 24-well tissue culture plates in complete medium and were allowed to adhere for 4 h prior to addition of toxicants ($ZnCl_2$, $CdCl_2$, CDDP, or *t*BH). Cells were grown for two days, after which the culture medium was removed and the cells were washed twice with PBS. The cells were trypsinized and the number of cells measured by direct counting (Coulter Counter Z_1). Relative survival (RCS) was expressed as:

$$\text{RCS} = \frac{[\text{number of cells treated with toxic agent}]}{[\text{number of cells treated with vehicle alone}]} \times 100$$

Over 98% of adherent cells measured at the end of the exposure period were viable (assessed by Trypan Blue dye exclusion). Reduced cell number after two days exposure to toxicants was due to loss of adherence and release into culture medium of a fraction of exposed cells (data not shown) and/or inhibition of proliferation of viable adherent cells.

Zn-induced resistance to toxicants. Cells were pretreated with 80 µM $ZnCl_2$ for 24 h prior to seeding. The cells were then trypsinized, counted, seeded into 24-well tissue culture plates, and resistance to Zn treatment assessed as described above.

Statistical Analysis

All values are presented as the mean of n measurements ± standard error (SE). Differences among means for corresponding sets of data were evaluated using one way ANOVA followed by a *post hoc* Tukey's multiple comparison test. The level of significance for all statistical analyses was chosen *a priori* to be $p \leq 0.05$.

RESULTS

Characterization of MT-Expressing and MT-Null Cell Lines

To determine whether the loss of MT-1 and MT-2 in the two MT-null cell lines (MT-KO1 and MT-KO2) was associated with altered, compensatory expression of genes involved in cellular stresses, we determined the level of expression of heme oxygenase-1 (HO-1), gamma-glutamyl-cysteine synthetase (γ-GCS) and Cu/Zn superoxide dismutase (SOD-1), relative to GAPDH, in MT-WT, MT-KO1, and MT-KO2 cells. We have reported previously that the MT knockout does not affect expression of either MT-3 or MT-4 (Butcher *et al.*, 2004). In the present investigation, however, HO-1 and SOD-1 mRNA levels were significantly greater in MT-KO2 cells than in either the MT-WT or MT-KO1 cell lines. By contrast, levels of γ-GCS mRNA were significantly lower in MT-KO1 and MT-KO2 cells than in the MT-WT cells (Fig. 1).

Because MTs have been associated with proliferation (Haq *et al.*, 2003), growth rates of all cell lines were assessed to determine whether loss of MT-1 and/or MT-2 affected proliferation in tissue culture. All three cell lines, regardless of MT status, had similar proliferation rates with doubling times of 20.0 ± 1.5 h (MT-WT), 19.0 ± 0.9 h (MT-KO1), and 16.1 ± 1.1 h (MT-KO2) (Fig. 2). There were no significant differences among the doubling times ($p = 0.133$, one way ANOVA).

Basal and Zn-Induced Resistance to Toxicity

Numerous studies have assessed the role of MT, in the context of Zn pre-treatment to induce elevated levels of MT protein for providing protection against toxicants. In those studies the induced MT was correlated with the enhanced resistance or level of protection afforded to a variety of toxic events (Notta *et al.*, in press). However, since Zn-pretreatment can apparently stimulate resistance events other than those mediated by MT, we determined whether enhanced resistance to Cd, Zn, CDDP, and *t*BH was mediated by Zn-induced MT or other Zn-induced events; by comparing the level of Zn-induced resistance to these toxicants between MT-null and MT-competent cells.

Resistance of MT-Null and MT-Positive Cell Lines to Cd

Inhibition of cellular proliferation mediated by exposure to Cd was assessed in the MT-WT, MT-KO1, and MT-KO2 cell

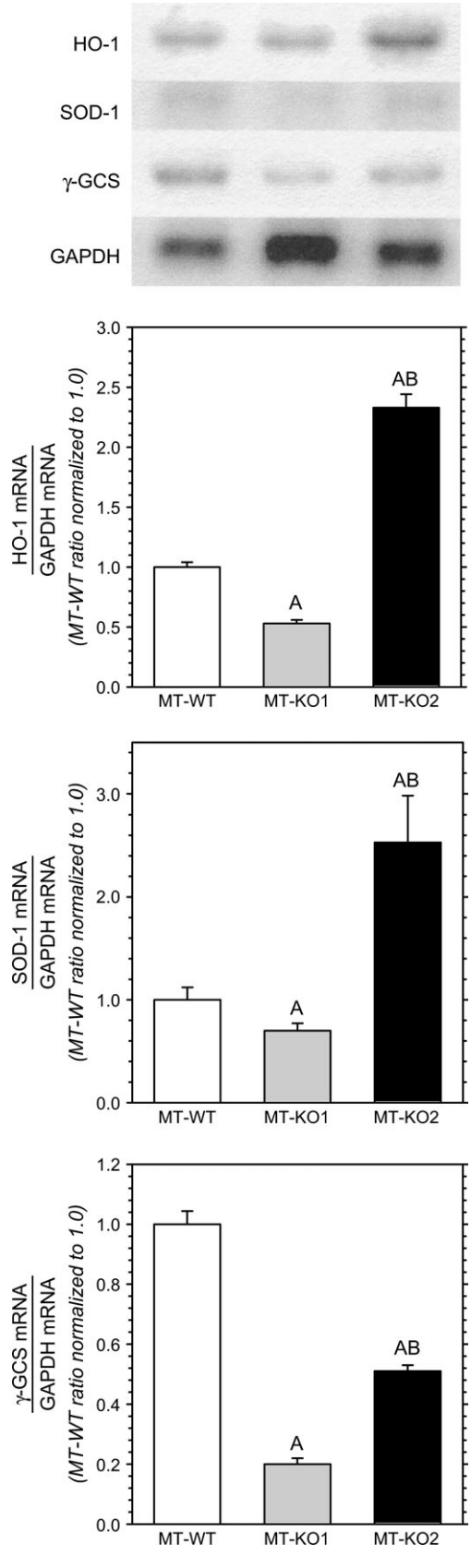

FIG. 1. Basal levels of HO-1 mRNA (1600 bp transcript), SOD-1 mRNA (470 bp transcript), and γGCS mRNA (1914 bp transcript) in MT-WT and MT-KO cell lines. Representative triplicate blots of total cellular RNA isolated from independent cultures of each cell line are shown, with graphic presentation of mean values ± SE. A: Significantly different from MT-WT (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$). B: Significantly different from MT-KO1 (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$).

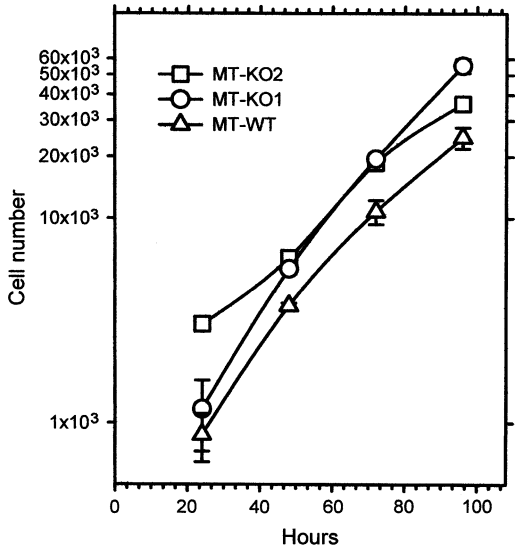

FIG. 2. Growth kinetics of MT-WT, MT-KO1, and MT-KO2 cells. Data points indicate the mean of three independent measurements ± SE. Where error bars are not visible they are smaller than the symbol. Doubling times, calculated from the linear portion of the curves between 48 and 72 h growth, were: MT-WT (20.0 ± 1.5 h), MT-KO1 (19.0 ± 0.9 h), and MT-KO2 (16.1 ± 1.1 h). There were no significant differences among the doubling times ($p = 0.133$, one-way ANOVA).

lines. Cellular growth was measured in these cell lines two days after exposure to 1.0–2.0 μM $CdCl_2$. Surprisingly, both MT-KO1 and MT-KO2 cell lines were more resistant to the toxic effects of all Cd levels than the MT-positive cell line, with MT-KO2 cells demonstrating a greater level of resistance than the MT-KO1 cells (Figs. 3A and 3B). After inducing MT, by exposing cells to 80 μM $ZnCl_2$ for 24 h, MT-WT cells exhibited a 2.5-fold, 3.4-fold, and 3.6-fold increase in resistance to treatment with 1.0 μM, 1.25 μM, or 2.0 μM $CdCl_2$, respectively. Neither MT-KO1 nor MT-KO2 cells that were pretreated with Zn exhibited an induced resistance to treatment with Cd (Fig. 3C).

Resistance of MT-Null and MT-Positive Cell Lines to CDDP

Sensitivity of MT-WT, MT-KO1, and MT-KO2 cells to CDDP was assessed by measuring cell numbers and calculating CDDP-induced inhibition of growth following two days of CDDP treatment. Survival for each cell line was evaluated after exposure to increasing concentrations of CDDP.

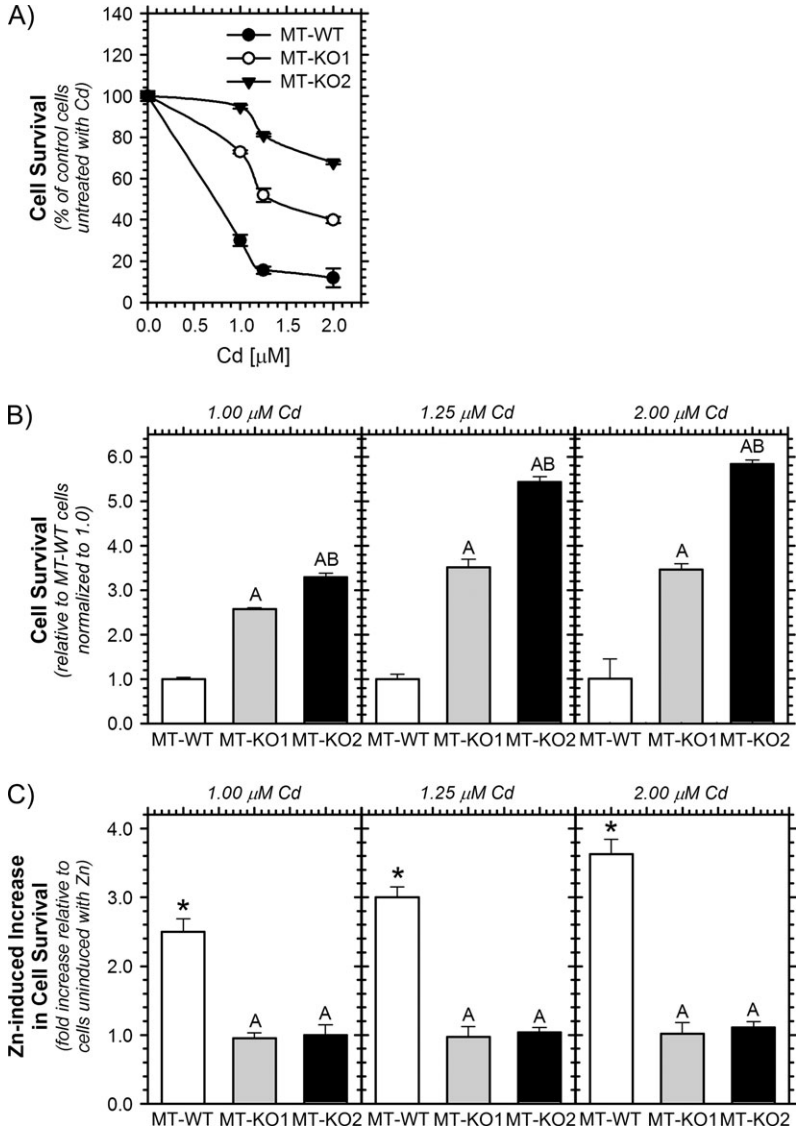

FIG. 3. Sensitivity of MT-WT cells (open bars), MT-KO1 cells (shaded bars), and MT-KO2 cells (black bars) to Cd before and after induction with Zn. Independent triplicate cultures were exposed to the indicated concentrations of $CdCl_2$ before or after induction for 24 h with 100 μM $ZnCl_2$. (A) Cell survival assessed as the number of cells after 48 h growth in Cd. To show dose-dependent decreases in cell survival, sensitivity to Cd relative to control cells unexposed to Cd and without prior Zn induction is shown. (B) To show differences between MT-KO and MT-WT cells uninduced with Zn, sensitivity to Cd is shown relative to values for MT-WT cells normalized to 1.0. (C) To show Zn-induced changes in Cd sensitivity, values for cells pretreated with $ZnCl_2$ are shown relative to values for parallel cell cultures uninduced with Zn. A: Significantly different from MT-WT (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$). B: Significantly different from MT-KO1 (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$). *Significantly different from cells uninduced with Zn (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$).

Consistent with the sensitivity of MT-WT and MT-null cells to Cd, MT-WT cells were more sensitive to CDDP than either of the other two MT-null cell lines, particularly at CDDP concentrations greater than 0.1 μM (Figs. 4A and 4B). MT-KO1 cells were more resistant to CDDP than MT-KO2 cells (opposite to the relative resistance of the two MT-null cell lines to Cd). Consistent with Zn-induced resistance to Cd, however, induction of MT-WT cells with 80 μM $ZnCl_2$ for 24 h resulted in a 1.5-fold and a 1.6-fold increase in resistance to treatment with 0.65 μM and 1.65 μM CDDP, respectively. Pretreatment with Zn did not increase resistance to CDDP in either of the two MT-null cell lines (Fig. 4C).

Resistance of MT-Null and MT-Positive Cell Lines to Toxic Effects of tBH

Sensitivity to *tert*-butyl hydroperoxide (*t*BH) was assessed in MT-WT, MT-KO1, and MT-KO2 cell lines by measuring *t*BH-mediated inhibition of cellular growth in the presence or absence

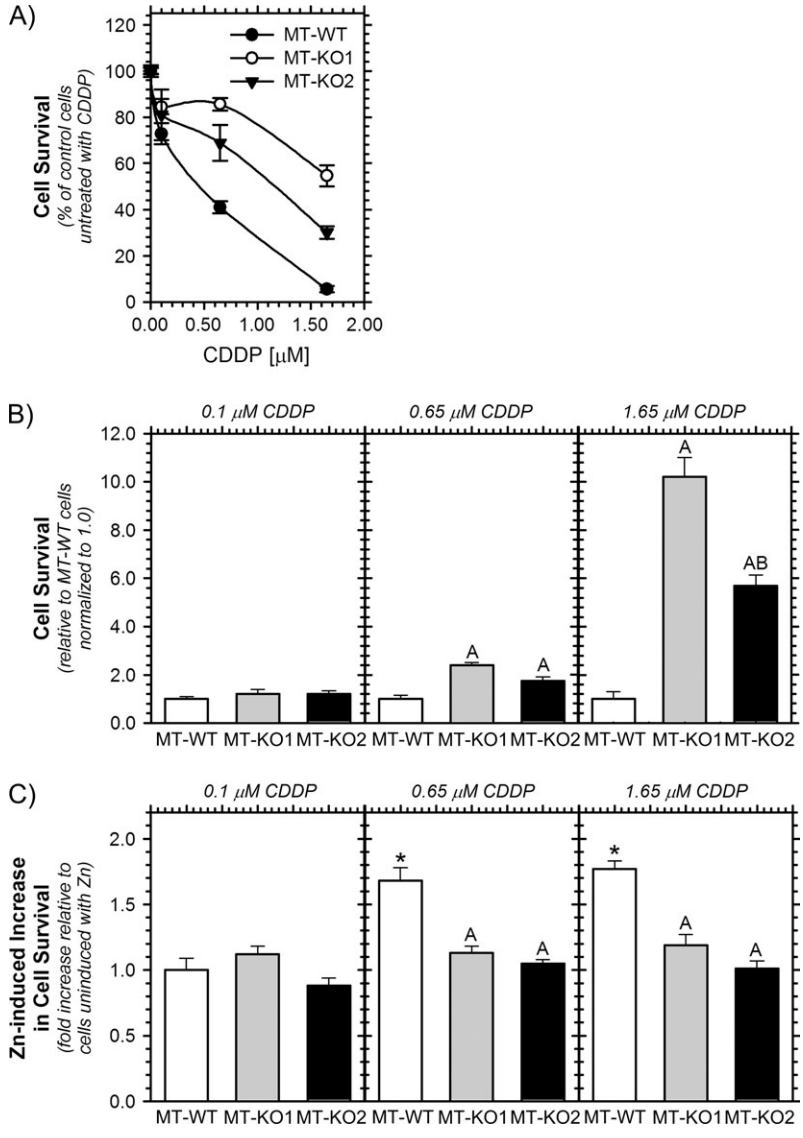

FIG. 4. Sensitivity of MT-WT cells (open bars), MT-KO1 cells (shaded bars), and MT-KO2 cells (black bars) to CDDP (CDDP) before and after induction with Zn. Each cell line was exposed, in independent triplicate cultures, to various concentrations of CDDP before or after induction for 24 h with 100 μM $ZnCl_2$. Data are presented as described in the legend to Figure 3.

of Zn pretreatment. Survival in each of the cell lines was measured after exposure to increasing concentrations of *t*BH over a two-day period. Contrary to the resistance profile of MT-WT and MT-null cells detected with Cd or CDDP, MT-WT cells were more resistant to *t*BH than MT-null cells (Figs. 5A and 5B). After pretreatment with 80 μM $ZnCl_2$ for 24 h, there was no significant change in resistance to *t*BH in MT-WT cells: in fact, Zn pretreatment caused resistance to *t*BH to decrease by 20, 40, and 43% following two days of exposure to 10 μM, 20 μM, and 25 μM *t*BH, respectively (Fig. 5C). After Zn-pretreatment, resistance to the highest concentrations of *t*BH increased only in MT-KO2 cells, but not in the MT-KO1 cells, relative to the level of *t*BH resistance in Zn-pretreated MT-WT cells.

Resistance of MT-Null and MT-Positive Cell Lines to Zn

Sensitivity to Zn in the MT-WT, MT-KO1, and MT-KO2 cells was assessed by measuring Zn-mediated inhibition of growth, with or without pretreatment with 80 μM Zn, two days after secondary Zn exposure. Survival of each cell line was measured after exposure to increasing concentrations of Zn. MT-KO2 cells were slightly more resistant to the toxic effects of Zn than either MT-WT or MT-KO1 cells. There was no significant difference in the protection provided by Zn between MT-KO1 and MT-WT cells (Figs. 6A and 6B). All three cell lines demonstrated increased levels of resistance to the toxic effects of Zn following Zn pretreatment, with the greatest Zn-induced increases in resistance detected at the highest

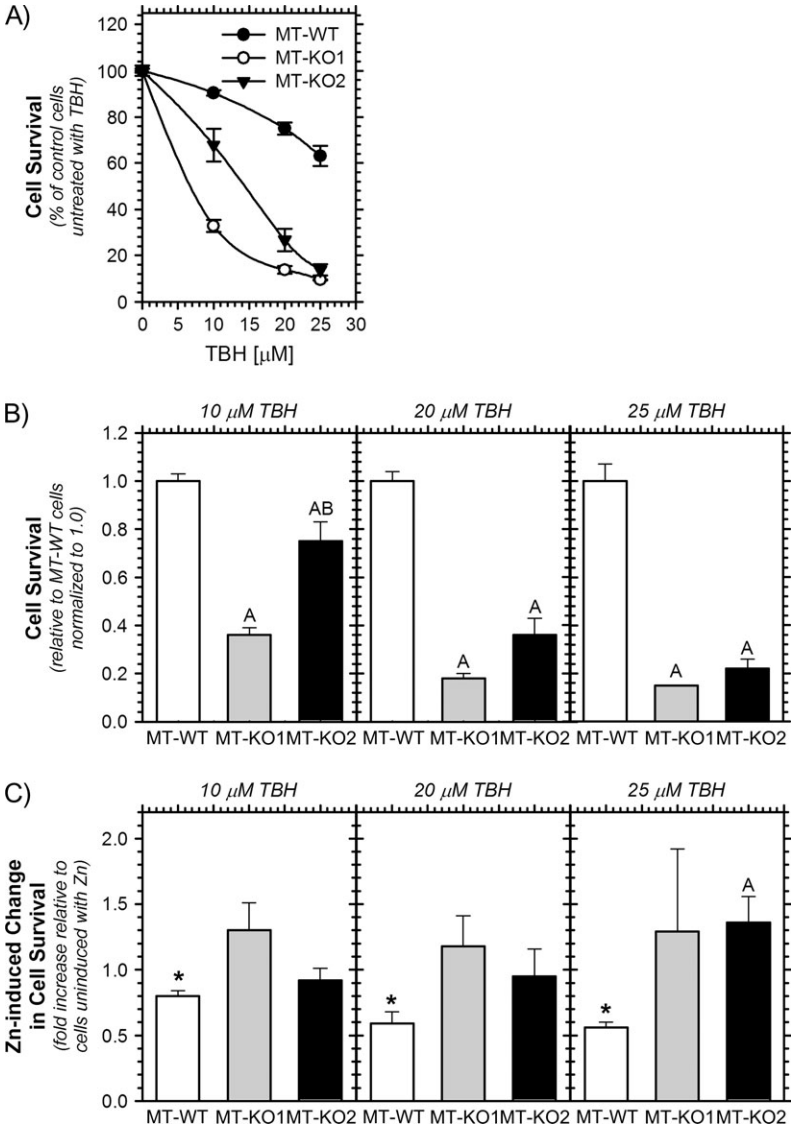

FIG. 5. Sensitivity of MT-WT cells (open bars), MT-KO1 cells (shaded bars), and MT-KO2 cells (black bars) to *tert*butylhydroperoxide (TBH) before and after induction with Zn. Each cell line was exposed, in independent triplicate cultures, to various concentrations of TBH before or after induction for 24 h with 100 μM $ZnCl_2$. Data are presented as described in the legend to Figure 3.

concentrations of secondary, Zn exposure. MT-WT cells showed a 4.0-fold increase, the MT-KO1 cells showed a 3.3-fold increase and MT-KO2 cells showed a 2.2-fold increase, in resistance to treatment with 140 μM $ZnCl_2$ (Fig. 6C).

Resistance of Antisense MT RNA-Expressing and Control HeLa Cells to Toxicity

Down-regulation of MT mRNA and protein by antisense treatment in three independent clonal transfectant populations of human HeLa cells (pNMH-asMT 4, 5, and 6) was used to investigate the role of MT in resistance to the toxic effects of Cd, CDDP, and *t*BH. The resistance of these three cell lines to Cd, CDDP, and *t*BH was compared to five independent control cell lines transfected with vector alone (pNMH4, 5, 6, 7, and 8).

MT mRNA and Protein Levels in HeLa Cell Lines

Expression of antisense mouse MT-1 RNA in three human HeLa cell clones (pNMH-asMT4, pNMH-asMT5, and pNMH-asMT6) transfected independently with the pNMH-asMT-1 expression vector was confirmed by detection of amplified mouse MT-1 cDNA by RT-PCR. Lack of antisense MT-1 RNA in five HeLa cell clones (pNMH4, pNMH5, pNMH6, pNMH7, and pNMH8) was similarly confirmed by the absence amplified mouse MT-1 cDNA product (Fig. 7). MT protein was assessed in all control and antisense mouse MT-1-expressing clones by

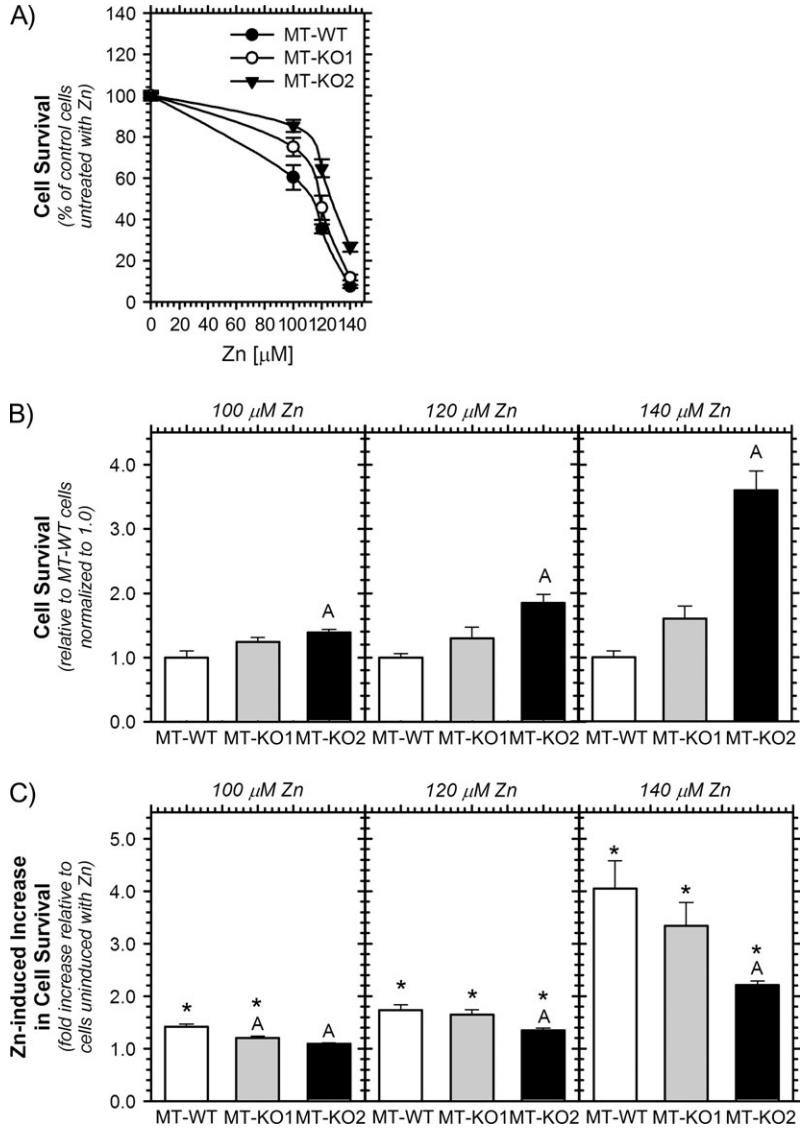

FIG. 6. Sensitivity of MT-WT cells (open bars), MT-KO1 cells (shaded bars), and MT-KO2 cells (black bars) to $ZnCl_2$ before and after induction with Zn. Each cell line was exposed, in independent triplicate cultures, to various concentrations of $ZnCl_2$ before or after induction for 24 h with 100 μM $ZnCl_2$. Data are presented as described in the legend to Figure 3.

the competitive DELFIA for MT. All three antisense MT RNA-expressing clones had lower MT protein levels than the control HeLa cell clone containing the lowest level of MT.

Effect of Antisense-Downregulation of MT on Resistance of HeLa Cells to Cd, CDDP, or tBH

Sensitivity to Cd, CDDP, and *t*BH of the three HeLa clones with antisense-mediated decreases in MT, and the five control clones with higher, basal MT, was assessed by measuring inhibition of growth over a two-day period after the addition of $CdCl_2$ (1.0–4.0 μM), CDDP (0.2–2.0 μM), or *t*BH (10–25 μM). There was no effect of antisense downregulation of MT on Cd or CDDP resistance at any concentration of either of the two toxic agents. However, antisense-mediated reduction in MT decreased resistance to *t*BH at the two highest tested concentrations (20 and 25 μM *t*BH). All three antisense MT-expressing clones with reduced basal MT were more sensitive to *t*BH at these concentrations than every control HeLa cell clone (Fig. 8).

DISCUSSION

Expression of MT correlates with a broad range of events, including resistance to heavy metals and other toxicants; cellular proliferation, differentiation and hypertrophy; and innate immune function. Given their importance in normal

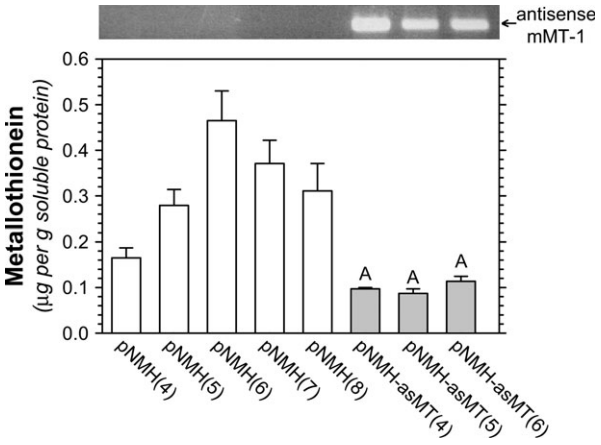

FIG. 7. Downregulation of MT in human HeLa cells by antisense MT RNA expression. Independent cultures of HeLa cells were stably transfected with an empty pNMH vector to generate five control HeLa populations [pNMH(4), pNMH(5), pNMH(6), pNMH(7), and pNMH(8), open bars]. Three HeLa cell populations expressing antisense mouse MT-1 cDNA from a pNMH-asMT vector were similarly generated [pNMH-asMT(4), pNMH-asMT(5), and pNMH-asMT(6), shaded bars]. Expression of antisense MT RNA was confirmed by gel electrophoresis and ethidium bromide staining of RT-PCR product from total cellular RNA, generated using primers specific for antisense MT-1 RNA (and not MT-1 mRNA) in the initial cDNA synthesis step (top blot). MT protein concentrations were measured in each control and asMT HeLa population by competitive ELISA for MT, in triplicate. Each bar represents the mean ± SE. A: Significantly different from every control HeLa cell population containing an empty pNMH vector (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$).

development and survival, MTs have been hypothesized to be essential for sustaining life. However, MT-1/MT-2 knockout mice develop normally and the only major negative consequence of MT-1 and/or MT-2 gene ablation is increased sensitivity to certain toxicants (Masters *et al.*, 1994). Therefore, the only physiological role for MT-1/MT-2, for which there is direct *in vivo* evidence, is in resistance to heavy metal intoxication and other toxic stresses, particularly oxidative stress. Because MTs bind metals and reactive oxygen species directly (Thornalley and Vasak, 1985), it has been hypothesized that the mechanism by which MTs mediate resistance is by direct inactivation of damaging species to spare sensitive cellular macromolecules and structures from damage (Cherian *et al.*, 2003). However, evidence is mounting to support the view that MTs regulate metalloproteins important in multiple physiological events, including (but not restricted to) toxicant resistance. These metalloproteins include transcription factors and hormone receptors; such as NF-κB (Butcher *et al.*, 2004), p53 (Meplan *et al.*, 2000), MTF-1 (Bi *et al.*, 2004; Zhang *et al.*, 2003), and GR (DeMoor *et al.*, 2001; Vallee *et al.*, 1991; Vallee and Falchuk, 1993) possibly by homeostatic regulation of Zn (Koropatnick and Leibbrandt, 1995). Although MTs bind Zn with high affinity, they can donate or sequester it directly to and from Zn-requiring transcription factors (Vallee and Falchuk, 1993). In addition, Zn released from MTs after exposure to reactive oxygen species has been suggested to be protective in its own right (Powell, 2000), and can be used by Zn-requiring enzymes that protect from toxicants but are unable to acquire Zn directly from its high-affinity association with MT (Maret, 2003). Multiple cellular processes may, therefore, depend on MTs, and their ability to act in more than one fashion to influence different cellular events. This has implications for the effect of increased or decreased MT expression on toxicant sensitivity, since MT expression may differ in response to different challenges.

To address these issues, we investigated the role of basal and Zn-induced MT in resistance to a panel of toxicants (Cd, Zn, CDDP, and *t*BH) using renal sustentacular cells from control and MT-knockout mice. To assess intrinsic, non-MT effects in these cell lines (with the potential to affect toxicant sensitivity), we measured expression of genes implicated in cellular resistance to reactive oxygen and chemotherapeutic drugs (HO-1, SOD-1, and γ-GCS). High or low HO-1 or SOD-1 expression did not segregate with MT-1/MT-2 expression in MT-WT or MT-KO cells (Fig. 1) making them unlikely to account for differences in toxicant sensitivity. γ-GCS mRNA levels, on the other hand, were lower in both MT-KO cell lines compared to MT-WT. However, γ-GCS mediates the rate-limiting step in the synthesis of glutathione (GSH) and is positively correlated with cellular redox status and protection against oxidative stress, multiple cancer chemotherapeutic drugs, and ionizing radiation (Fojo and Bates, 2003). Lower basal γ-GCS mRNA levels in relatively Cd, CDDP, and Zn-resistant MT-KO cell lines was opposite to the pattern expected in view of these γ-GCS correlations. Within the limits of interpretation of mRNA levels alone without protein level and activity information, these data collectively suggest that HO-1, SOD-1, and γ-GCS are not consequential determinants of resistance to Cd, Zn, or CDDP in MT-WT or MT-KO cells.

Although MT expression has been correlated with proliferation, and primary MT-null T cells have been reported to have decreased mitogen-induced proliferation (Mita *et al.*, 2002), no effect of MT knockout on *in vitro* basal proliferative rate has been reported. Differences in rates of cellular growth have been associated with resistance to some drugs and toxicants (Peters *et al.*, 2002) and altered growth rate, if induced by MT knockout, might result in enhanced sensitivity to toxicants. We have not observed significant differences in proliferation among MT-WT and MT-KO cell lines (Fig. 2), suggesting that MT-dependent differences in toxicant-sensitivity are not due to altered growth.

When sensitivity of MT-WT and MT-KO cells to Cd or Zn was assessed, we were surprised to find that MT-WT cells were more sensitive to the toxic effects of Cd or Zn than either MT-KO1 and MT-KO2 cells (Figs. 3 and 6). We have reported previously that both MT-KO1 and MT-KO2 cell lines have decreased cytosolic levels of MT protein relative to MT-WT cells (Butcher *et al.*, 2004). Therefore, basal MT-1 and/or MT-2 do not appear to act as independent markers of resistance to the

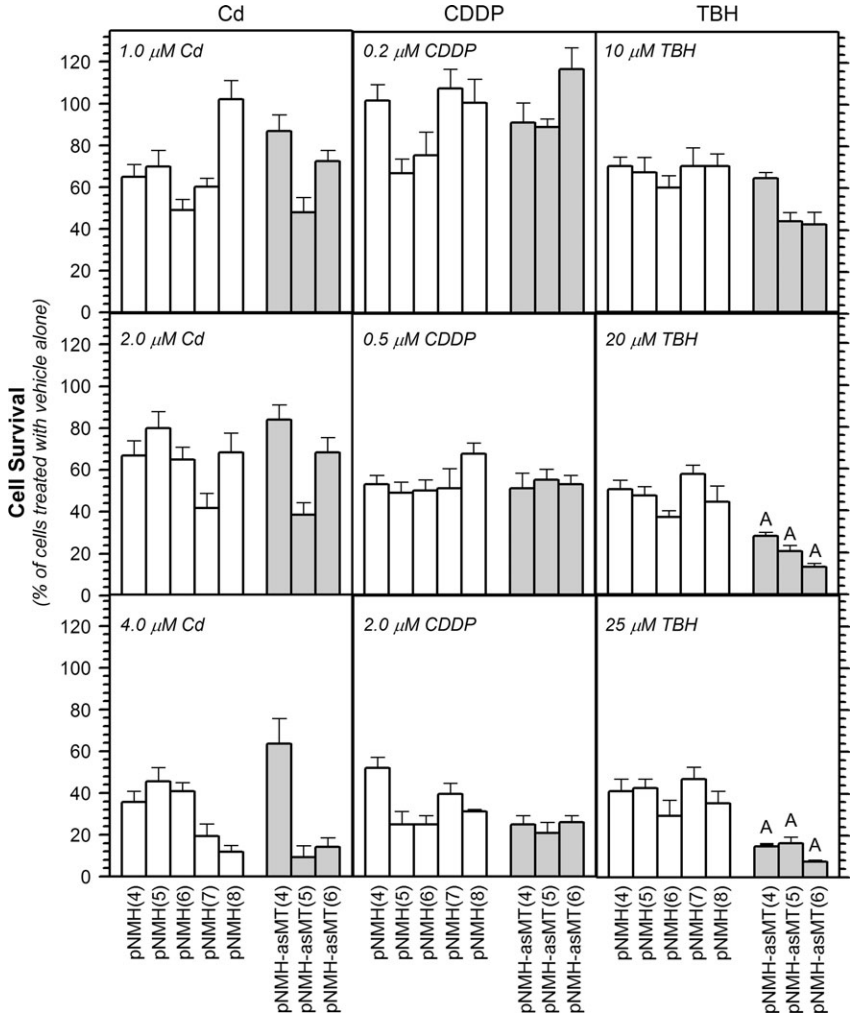

FIG. 8. Sensitivity of HeLa cells with normal control MT levels (five populations, open bars) and antisense-downregulated MT (three populations, shaded bars) to Cd, CDDP, or TBH. Cells were grown for two days with or without toxin treatment and the sensitivity to toxin calculated as the number of toxin-treated cells as a percentage of the number of cells after exposure to control vehicle alone. A: Significantly different from every control HeLa cell population (one-way ANOVA and *post hoc* Tukey's multiple comparison test, $p \leq 0.01$).

toxic effects of Cd or Zn. Known mediators of resistance to some toxins either did not consistently segregate with loss of MT-1 and MT-2 (HO-1 and SOD-1), or were lower in MT-KO1 and MT-KO2 in spite of resistance to Cd and Zn relative to MT-WT (Fig. 1). Other cellular characteristics, independent of MT (presently uncharacterized but potentially including capacity for metal uptake, activity of pro- and antiapoptotic enzymes and transcription factors, and others), appear to mediate differences in metal sensitivity and diminish or nullify the effect of the presence or absence of low basal MT levels.

Alternatively, loss of MT might have resulted in activation of other, non-MT cellular events mediating sensitivity to Cd or Zn or inactivation of events mediating resistance to these metals. Although we did not assess possible mechanisms underlying this relative resistance of MT-KO cell lines to Cd and Zn (compared to MT-WT cells), it is clear that basal MT-1 and/or MT-2 protein do not, alone, protect from the toxic effects of Cd or Zn. Other events, independent of MT, can apparently provide protection against these metal toxicants in MT-null cells.

On the other hand, Zn induction of MT enhanced resistance to Cd only in MT-WT cells, and not in either of the two MT-KO cell lines lacking basal or Zn-inducible MT expression. In view of our previous report that pretreatment with Zn increases MT protein only in MT-WT and not in either MT-KO1 or MT-KO2 cells (Butcher *et al.*, 2004), this clearly indicates that induced MT-1 and/or MT-2 are the critical factors mediating Zn-induced resistance to the toxic effects of Cd. Therefore, despite Zn mediating multiple cellular events (including induction of both MT and non-MT genes), Zn-induced non-MT events had no effect on Cd resistance. Although MT expression has been correlated with protection against Cd, to our knowledge, this is the first report that MTs, and not non-MT factors, are the

critical molecules induced by Zn pretreatment that mediate Cd resistance.

Contrary to the effects of Zn-exposure on the sensitivity to the toxic effects of Cd, pretreatment with Zn resulted in enhanced resistance to subsequent exposure to toxic levels of Zn in all cell lines, regardless of the presence or absence of Zn-inducible MT genes. Therefore, neither basal nor induced MT appeared to be a critical factor mediating Zn resistance. Although Zn is rarely considered to be toxic except under exceptional circumstances, zinc overload may be an important factor in neurodegenerative disease states (Dineley *et al.*, 2003). Data suggest that efforts to induce MT in neural tissues may be a useful approach to ameliorate or prevent such toxicity, but that induced MT is not likely to be a critical factor in mediating the protections afforded by Zn.

The pattern of basal and Zn-induced protection against CDDP was qualitatively similar to that against Cd in MT-WT and MT-KO cells (Fig. 4). Therefore, basal MT does not appear to necessarily mediate CDDP resistance, which is in accord with reports implicating factors other than MT in providing protection (Akiyama *et al.*, 1999). On the other hand, Zn induction of MT did enhance cellular resistance to CDDP, but only in MT-WT cells, similar to the situation detected with Cd. These observations suggest that differences in MT expression at low levels may not correlate with resistance to the toxic effects of CDDP, but that differences at higher levels (in the range induced by Zn) may.

A very different pattern of sensitivity to *t*BH was observed. Contrary to the situation detected with Cd, Zn, and CDDP, MT-WT cells were more resistant to *t*BH than either MT-KO cell line, suggesting that low, basal MT had the capacity to mediate resistance (Fig. 5). γ-GCS levels were also lower in both MT-KO cell lines, suggesting a role for this difference (possibly induced by MT gene ablation) that is currently under investigation in our laboratory. This effect of differences in MT in mouse fibroblasts was paralleled in the human HeLa cells; where antisense-mediated reduction in MT reduced resistance to *t*BH (but not resistance to Cd or CDDP) in multiple, independent clonal populations compared with a panel of control HeLa clones (Fig. 6). When MT-KO cells were pretreated with Zn, there was no increase in resistance to *t*BH, in spite of increased MT expression. It appears that basal MT, reduced by different mechanisms in both mouse and human cells, plays a key role in mediating resistance to *t*BH, but not Cd, Zn, or CDDP. On the other hand, increased MT levels, resulting from Zn pretreatment, had little or no effect on *t*BH resistance. These observations are not consistent with the hypothesis that MT interacts directly with toxic ROS generated by *t*BH, where an increase in the level of MT would mediate an increase in resistance. Rather, it is in line with the concept that MTs may mediate the activity of one or more factor(s) important in *t*BH resistance: in the case of *t*BH, low levels of MT might be sufficient to ensure optimal or maximal activity of such factors. If the level of those factors is not increased by Zn induction, then increased MT levels induced by Zn pretreatment would not enhance factor-activity beyond the ''plateau level'' supported by basal MT expression. On the other hand, Zn-induced resistance to Cd and CDDP is consistent with, a role for MT in directly interacting with and inactivating these toxicants or, damaging species induced in cells by exposure to them, without ruling out the possibility that MT might mediate the function of MT-dependent resistance factors at the same time.

Overall, our data support the contention that, at low basal MT levels, factors other than MT can provide relative resistance to the toxic effects of some toxicants (Cd, CDDP, and Zn), but not others (*t*BH). This is exemplified in the mouse cells lacking MT, as a result of gene knockout, and in the human cells with antisense-downregulated MT-expression. By contrast, elevated MT induced by Zn pretreatment, among other Zn-inducible factors, is a key factor in affording protection to the toxic effects of some agents (Cd and CDDP), but not others (Zn and *t*BH). Pretreatment with Zn induces cellular events, other than MT, which mediate increased resistance to subsequent exposure to potentially toxic concentrations of Zn, although induced MT does not appear to be a critical factor in affording protection. However, basal MT levels are important in the protection afforded during exposure to *t*BH, while increased levels of MT are not. These findings support the hypothesis that MTs mediate resistance through both direct mechanisms (interaction with and inactivation of toxic species) and indirect mechanisms (possibly including mediating the activity of proteins important in resistance to toxicants). The value of MTs in predicting resistance to toxicants, and the importance of MT in mediating toxicant resistance, appears to differ depending on the toxicant being tested, and depends on whether MT levels are compared at relatively low or high levels, or if MT levels are basal or induced (by Zn).

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