Enhanced Apoptosis in Metallothionein Null Cells

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SUMMARY

Metallothioneins (MTs) are major intracellular, zinc-binding proteins with antioxidant properties. Mouse embryonic cells null for MT due to loss of functional MT I and II genes (MT−/−) were more susceptible to apoptotic death after exposure to *tert*-butyl hydroperoxide or the anti-cancer agents cytosine arabinoside, bleomycin, melphalan, and *cis*-dichlorodiammineplatinum(II) compared with wild-type mouse embryonic cells (MT+/+). We measured basal levels of the tumor suppressor protein p53 and the death effector protein Bax and found the basal levels of both proteins were higher in MT null cells compared with MT+/+ cells. After treatment with the DNA-damaging agent *cis*-dichlorodiammineplatinum(II), p53 protein levels were in-duced in both MT+/+ and MT−/− cells with MT null cells always maintaining the highest p53 levels. The elevated sensitivity to apoptosis was not restricted to embryonic cells. Primary pulmonary fibroblasts were isolated from distinct litters of MT null, heterozygous, and wild-type mice, and all had undetectable basal MT levels. Zinc exposure increased MT levels in the wild-type and heterozygous fibroblasts but not in the MT null fibroblasts. Consistent with the induced MT levels, we found MT+/+ and MT+/− embryonic cells were less sensitive to *cis*-dichlorodiammineplatinum(II)-induced apoptosis compared with MT−/− cells. Our results implicate MT as a stress-responsive factor that can regulate apoptotic engagement.

Controlled cell death is essential for the development and survival of multicellular organisms. Several gene products, such as Bax, facilitate the selective and controlled process of death termed apoptosis (1). Apoptosis is inhibited by a variety of proteins including the oncoprotein Bcl-2 and the antioxidants superoxide dismutase and glutathione peroxidase (2). Inappropriate apoptosis may precipitate many diseases including Alzheimer's, Huntington's, and Parkinson's diseases, neoplasia, autoimmune disorders, immune deficiency, ischemic neurological and cardiovascular damage, and alopecia (3). Because apoptosis is a critical homeostatic mechanism, it is likely to be finely regulated by both constitutive and inducible proteins. For example, the tumor suppressor p53, which is a zinc-dependent protein, seems to be one inducible stress-responsive protein that facilitates some pathways of apoptosis.

The morphological properties of apoptosis, most notably chromatin condensation, are now well accepted (4, 5). The biochemical bases for chromatin condensation and the other morphological aspects of apoptosis remain poorly defined. Some have suggested ROI may participate in apoptotic signaling (2, 6–9), whereas others have disputed this hypothesis (10, 11). DNA cleavage is universally seen in nucleated cells undergoing apoptosis. A Ca^{2+}/Mg^{2+}-dependent endonuclease activity that produces internucleosomal DNA breaks has been associated with the terminal phases of apoptosis in some cells, and this endonuclease is inhibited by zinc (12).

MTs are a family of low molecular weight thiol-rich proteins. As one of the major intracellular zinc-binding proteins, they may regulate free zinc levels. The intracellular levels of MT can be readily increased by heavy metals, cytokines, drugs, steroids, and low oxygen via transcriptional activation (13). Because of the nucleophilicity of MT, there has been considerable interest in its ability to protect cells against electrophilic toxins. Indeed, we and others (13–17) previously demonstrated increased MT protects against cytotoxicity from carbon-, oxygen-, and nitrogen-based radicals, including electrophilic mutagens, antineoplastic drugs, nitric oxide, and environmental oxidants. Cells made deficient in MT by homologous recombination and disruption of MT I and II genes are more sensitive to the toxic effect of oxidants, the heavy metal Cd, anti-cancer drugs, and electrophilic mutagens (18, 19). Zheng *et al.* (20) found increased hepatic induction of p53 in MT I and MT II knockout mice after Cd

ABBREVIATIONS: MT, metallothionein; ROI, reactive oxygen intermediates, ara-C, cytosine arabinoside; tBH, *tert*-butyl hydroperoxide; CDDP, *cis*-dichlorodiammineplatinum(II) (cisplatin); QFIGE, quantitative field inversion gel electrophoresis; PBS, phosphate-buffered saline; MEC, mouse embryonic cells; BLM, bleomycin.

exposure. A role for MT in apoptosis has not, however, been affirmed.

MT clearly can covalently interact and sequester electrophilic agents *in vitro* (13, 21), although the relative importance of this *in vivo* has not been established. Because ectopic expression of MT after transfection does not reduce DNA adduct formation seen with exposure to mutagens, other mechanisms may be operative (13, 15). Furthermore, MT seems to protect cells against antimetabolite anti-cancer drugs, such as cytosine arabinoside (ara-C) and tumor necrosis factor-α (13, 19, 22), an effect that cannot be readily rationalized by a hypothesis of simple covalent drug sequestration. Because of the great avidity of MT for zinc and its antioxidant capacity, we have directly investigated the potential role of this inducible protein to control apoptosis in mammalian cells. We report here that cells deficient in MT engaged apoptotic processes more readily than MT-replete cells. Our results illustrate the potential importance of the stress-inducible family of MT proteins in regulating the process of apoptosis.

Materials and Methods

Cells. Primary MEC derived from transgenic mice with disrupted MT I and II genes and from genetically matched mice with MT-proficient genes were provided by Drs. A. Michalska and A. H. K. Choo (Murdoch Institute for Research into Birth Defects, Melbourne, Australia). These MEC were established as a pooled population of cells from individual embryos isolated from 14.5-day pregnant females and were frozen at passage 6. The culturing conditions and biochemical characterization of these MEC have been reported previously (18). The thawed primary MT−/− and +/+ cells were never carried for more than 15 passages to avoid immortalization. No significant differences in responses were seen between passage 1 and 12 after thawing. The growth rates were similar between the two primary cell lines.

Inbred C57Bl/6 × Ola129 MT+/+, MT+/−, and MT−/− mice were maintained as previously described (23) with care to ensure maintenance of proper genetic background and virus-free mice. Primary pulmonary fibroblasts were isolated and maintained using the same methods we previously described for bovine and rabbit pulmonary fibroblasts (24). Three separate cell populations of each genotype were derived from three individual male mice that were age- and weight-matched and from different litters. Primary pulmonary fibroblasts were grown on plastic monolayer and were used within the first seven passages. No obvious morphological or growth differences were seen among the fibroblast populations. MT levels were determined using a Cd binding assay that has been described previously (19).

Morphological measurements of apoptosis. Primary embryonic cells were treated with vehicle or a concentration of oxidant or anti-cancer drug that caused 50% growth inhibition in wild type cells, i.e., 100 μM tBH, 25 μM bleomycin, 20 μM CDDP, 100 μM ara-C, or 30 μM melphalan. Drug exposure was for 48–72 hr, because initial studies with a 24-hr treatment revealed no significant drug-induced apoptosis (data not shown). Primary pulmonary fibroblasts that were either pretreated or not with 100 μM ZnCl$_2$ for 48 hr were exposed to 0–30 μM CDDP for 48 hr. Cells that were attached to the monolayer 48 or 72 hr after the treatment were harvested with a 3-min exposure to a PBS solution containing 0.25% trypsin and 2 mM EDTA and were combined with cells that detached from the monolayer after centrifugation at 1000 × g (5 min). The combined cells were resuspended in PBS containing 20 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 min at room temperature. Nuclei were visualized using a photomicroscope equipped with epifluorescence. We used Dunnett's or Student-Newman-Keuls multiple com-

parison *post hoc* tests for all studies to determine significant differences using a criteria of $p < 0.05$ as previously described (25).

DNA damage assays. MEC were treated and harvested as above. Attached and detached cells were combined, and both total and fragmented DNA, which remained in the 27,000 × g (20 min) supernatant fraction, were determined by Hoechst 33258 staining as described previously (25). Cells were washed three times with PBS and incubated with fresh medium.

DNA damage was also assessed by our previously described *in situ* break extension assay (25). Exponentially growing MT−/− or MT+/+ MEC were exposed to agents for 4 hr and incubated for an additional 3.75 hr. Cells were washed three times with PBS at 4° and fixed with 1% formaldehyde/PBS at 4° for 15 min. Cells were then permeabilized, and damaged DNA was labeled with 10 μM fluorescein-12-dUTP and 200 units/ml terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA). Nuclear fluorescence intensity in 100–200 cells was measured and analyzed with a Meridian ACAS 570c laser-scanning confocal microscope as previously described (25). In addition, the fraction of cells with nuclear fluorescence above the mean control value of 600 arbitrary fluorescence units was determined in a population of between 200 and 400 cells.

Conventional and field inversion gel electrophoresis. Low-molecular-weight internucleosomal DNA damage was assessed by conventional 1.8% agarose gel electrophoresis 24 hr after treatment as described previously (25). High-molecular-weight DNA fragments were measured by our previously described QFIGE method 24 hr after treatment (25).

Immunoblotting. Exponentially growing MEC (1 × 10^7 cells) were treated for 48 hr with 0–5 μM CDDP. In other studies we isolated exponentially growing primary pulmonary fibroblasts (MT+/+ and MT −/−) treated for 48 hr with 0 or 100 μM ZnCl$_2$. Cells were washed three times with PBS and then harvested by scraping in standard sodium dodecyl sulfide sample loading buffer (25, 26). Protein samples (20 μg) were prepared and immunoblotted with commercially available antibodies to: p53 [Ab-1 (Oncogene Science, Uniondale, NY) or CM5p (Novo Castra Labs, Newcastle upon Tyne, UK)], Bcl-2 (4C11) (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (P19), or Bcl-x (S-18) (Santa Cruz Biotechnology, Santa Cruz, CA) (5 μg/ml). Proteins were detected with a horseradish peroxidase-mediated luminal oxidase chemiluminescence method (Renaissance; Du-Pont NEN, Wilmington, DE). We performed at least three independent immunoblots with similar results for all reported experiments.
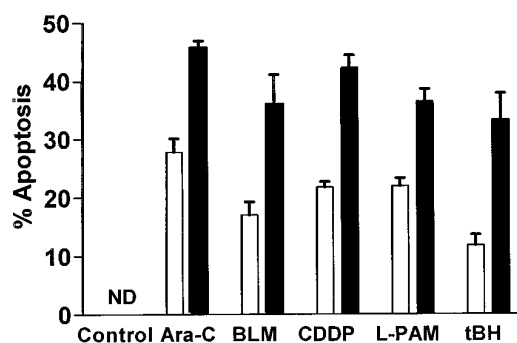
Results

We demonstrated previously that MT−/− MEC were more sensitive to growth inhibition by the heavy metal cadmium, by prototype and environmental oxidants, such as tBH and paraquat, and by several anti-cancer and mutagenic agents (18, 19). This enhanced sensitivity to growth inhibition could reflect either increased growth inhibition (i.e., cytostasis) or cell death (i.e., necrosis or apoptosis). Thus, we examined whether MT null cells had elevated apoptotic frequency before and after exposure to injurious agents. MT replete and null cells were exposed to concentrations of injurious agents that caused 50% growth inhibition (19), and cells were evaluated for apoptotic death after staining of DNA with Hoechst 33342. The spontaneous apoptotic levels in both MT+/+ and MT−/− MEC were <1%. Treatment of cells with the prototypic oxidant, tBH, or with anti-cancer agents produced frank morphological evidence of apoptosis. As illustrated in Fig. 1, exposure to 100 μM ara-C resulted in morphologically evident apoptosis in both MT+/+ and MT−/− MEC within 48 hr. We found no obvious qualitative difference in the apoptotic appearance of either population of cells after treatment with all of the injurious agents. Approximately 15–28%
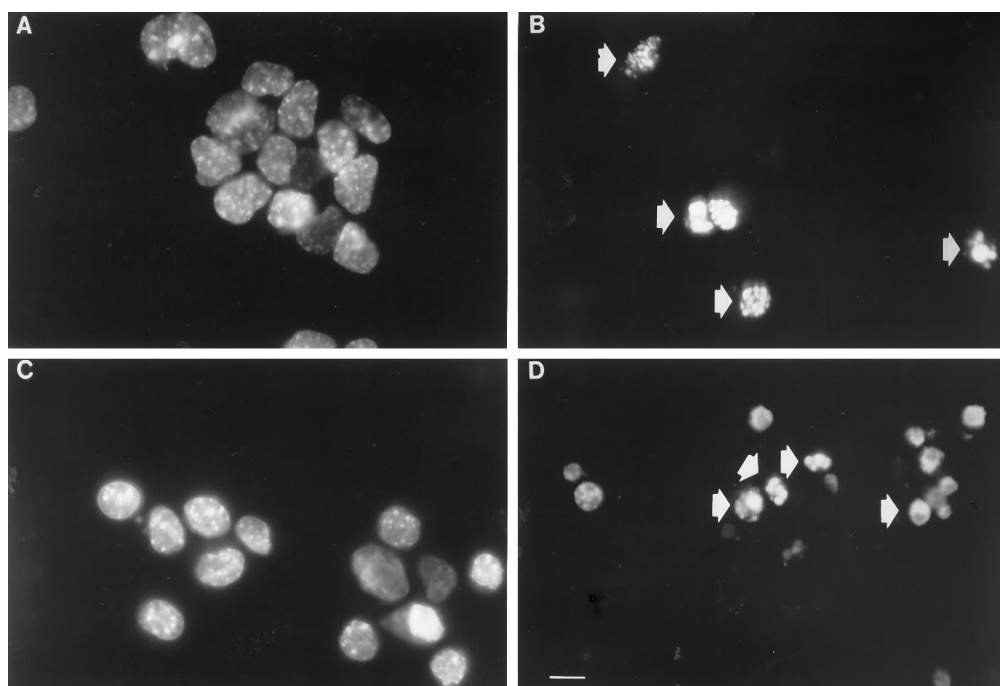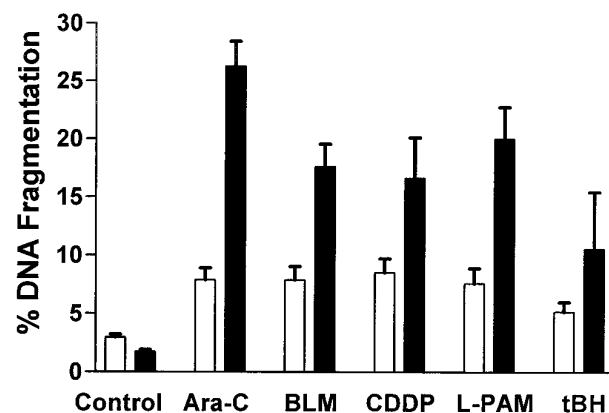
Fig. 1. Apoptotic morphology of MT−/− and MT+/+ MEC after treatment with ara-C. MT−/− (A and B) and MT+/+ (C and D) cells were untreated (A and C) or treated for 48 hr with 100 μM ara-C (B and D). Attached cells were harvested with trypsin and EDTA, combined with cells that detached from the monolayer, centrifuged, and resuspended in PBS containing Hoescht 33342. Nuclei were visualized using epifluorescence microscope. *Arrows*, typical apoptotic morphology. *Bar*, 10 μm.

of MT+/+ MEC had an apoptotic appearance 48 hr after exposure to the anti-cancer agents, ara-C, bleomycin, CDDP, and melphalan, or the oxidant tBH (Fig. 2). Treatment of MT−/− cells with these identical agents produced significantly more apoptosis (from 30 to 45%) than in MT+/+ cells (Fig. 2). Similar differences between MT−/− and MT+/+ MEC were also seen 72 hr after exposure to all of these agents (data not shown).

In nucleated cells apoptosis is universally accompanied by a loss of DNA integrity. Consistent with the increased morphological apoptotic appearance, DNA damage as assessed by the release of soluble DNA was significantly greater in MT−/− MEC than in MT+/+ cells after treatment with the injurious agents (Fig. 3). MT+/+ cells had approximately 5–10% of the total DNA in the soluble, low molecular weight DNA fraction compared with 16–25% in MT−/− cells 72 hr after treatment with similar concentrations of ara-C, bleomycin, CDDP, and melphalan (Fig. 3). There was no statis-
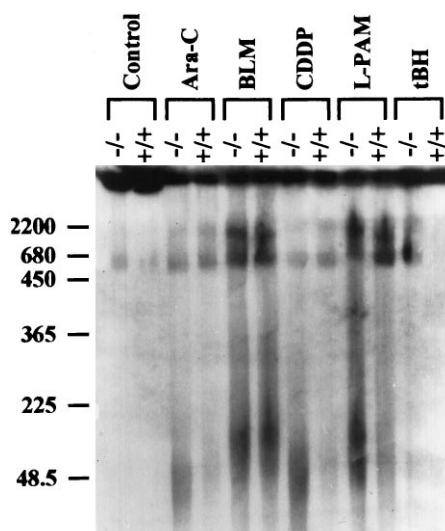

Fig. 3. DNA fragmentation of MT−/− and MT+/+ MEC after treatment with apoptotic agents. Cells were treated with injurious agents for 72 hr, and the soluble DNA was determined as described in Materials and Methods. All comparisons between drug-treated MT−/− and MT+/+ cells (except tBH) were statistically different ($p < 0.05$). □, MT+/+; ■ MT−/− cells ($n = 3$). *Bars,* mean ± standard error.

tically significant difference in the level of DNA damage detected by this method between MT+/+ and MT−/− cells treated with tBH (Fig. 3). The DNA damage profile seen after a 48-hr exposure to ara-C, bleomycin, CDDP, and tBH was similar to the 72-hr treatment, whereas after 24 hr there was little DNA damage and no significant difference between MT−/− and MT+/+ cells with any of the agents (data not shown). Internucleosomal DNA fragmentation in MT wild-type or null MEC was absent, regardless of the injurious agent used to treat cells, consistent with previous studies with MEC (26). The soluble, low molecular weight DNA damage appeared, therefore, to be higher order damage. QFIGE analysis indicated no significant DNA fragments of <450 kb in the untreated MT−/− and MT+/+ MEC but we did note DNA fragments of 500–800 kb, and there was a reproducibly greater amount in the MT−/− cells (Fig. 4). Injurious agents caused significantly greater higher order DNA damage, i.e.,

Fig. 2. Quantitative analysis of apoptotic morphology of MT−/− and MT+/+ MEC after treatment with apoptotic agents. Cells were exposed to injurious agents for 48 hr as described in Materials and Methods, and the apoptotic morphology was determined after staining with (20 μg/ml) Hoescht 33342. □, MT+/+; ■, MT−/−. $n = 3$. *Bars,* mean ± standard error. *ND*, none detected. All comparisons between drug-treated MT−/− and MT+/+ cells were statistically different ($p < 0.05$).
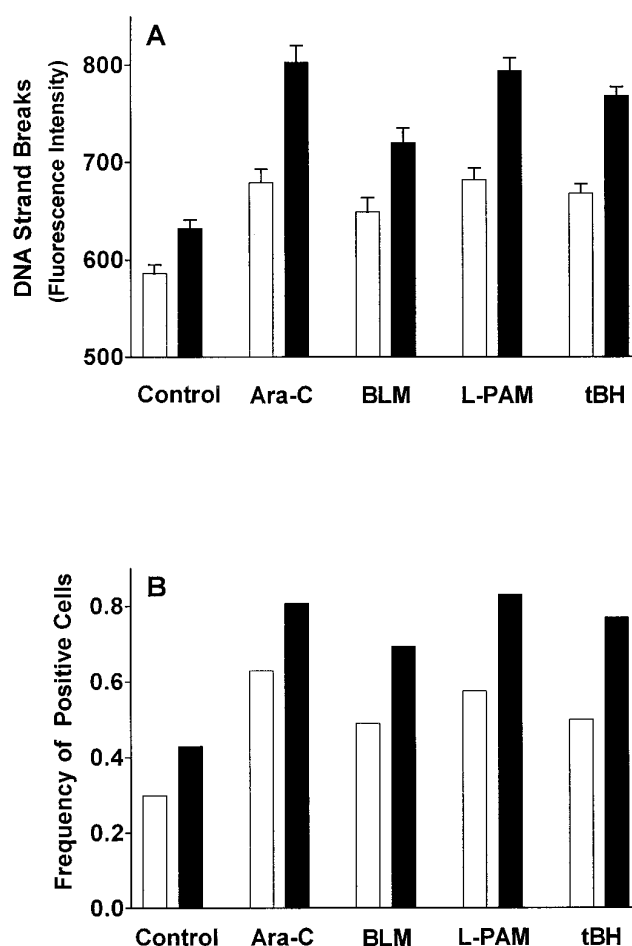
Fig. 4. High-molecular-weight DNA fragmentation. MT−/− and MT+/+ MEC were exposed to 100 μM tBH, 25 μM bleomycin, 20 μM CDDP, 100 μM ara-C, or 30 μM melphalan for 72 hr. Cells were harvested, and DNA was extracted and separated by field inversion gel electrophoresis. After Southern blot transfer and probing with a ^{32}P-labeled *Alu*I oligomer, the band intensity was visualized by PhosphorImager analysis as previously described (25). *Left,* migration of the DNA size markers indicated in kilobase pairs.

Fig. 5. *In situ* break extension assay in MT−/− and +/+ MEC after treatment with apoptotic agents. Cells were treated with 100 μM tBH, 25 μM bleomycin, 100 μM ara-C, or 30 μM melphalan for 72 hr and examined for DNA damage after labeling damaged DNA with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. □, MT+/+; ■, MT−/−. A, Nuclear fluorescence intensity in arbitrary units detected in 100–200 treated or untreated cells. All comparisons between drug-treated MT−/− and MT+/+ cells were statistically different ($p < 0.05$). *Bars,* mean ± standard error. B, Percent of the total population of cells with nuclear fluorescence >600 arbitrary units. Between 200 and 400 cells were counted.

30–50 and 500–800 kb, in both MT wild-type and null MEC. MT−/− cells had greater higher order DNA damage than MT+/+ cells after treatment with ara-C, CDDP, melphalan, and tBH (Fig. 4). Bleomycin produced prominent DNA fragments in both MT wild-type and null cells, presumably reflecting its ability to cause direct DNA damage.

The enhanced apoptosis in MT null cells was further confirmed using the terminal transferase assay, which suggested slightly greater basal DNA damage in MT−/− compared with MT+/+ MEC (Fig. 5, A and B) and which was in agreement with the slightly greater basal higher order DNA damage seen using QFIGE (Fig. 4). Consistent with both morphological and soluble DNA results, significant differences were seen in the DNA damage detected at 4 hr by terminal transferase in the MT wild-type and null cells after exposure to injurious agents (Fig. 5). Thus, using either total fluorescence intensity (Fig. 5A) or the percentage of labeled cells (Fig. 5B) as an index, MT null cells had markedly elevated DNA strand breaks after tBH, bleomycin, ara-C, and melphalan. MT+/+ cells also exhibited increased DNA strand breaks after treatment with these agents, but the level of damage was significantly less than that seen in MT−/− MEC.

To ensure the elevated levels of apoptosis were not due to clonal variation in the derived MEC or founder effects in the mice initially providing the MEC, we isolated pulmonary fibroblasts from mice of distinct litters. We previously found marked difference in the basal MT levels in MT+/+ and MT−/− MEC (18). In contrast, cultured pulmonary fibroblasts from MT+/+ mice had MT levels that were below the detection limits of our assay. Only after a 48-hr exposure to an inducer, such as ZnCl$_2$ (100 μM), were we able to detect MT in the MT+/+ cells (0.53 ± 0.09 μg/mg protein) and MT+/− cells (0.36 ± 0.02 μg/mg of protein); pulmonary fibroblasts from MT−/− mice had no detectable MT even after

pretreatment with ZnCl$_2$. Consistent with the induced levels of MT, we found MT+/+ and MT+/− cells were much less sensitive to CDDP-induced apoptosis compared with MT−/− cells (Fig. 6). CDDP-induced apoptosis in non-ZnCl$_2$-pretreated primary pulmonary fibroblasts was similar with each genotype, presumably reflecting the low levels of MT in all cells (Fig. 6).

Previously we observed that, compared with MT +/+ MEC, MT null MEC expressed higher basal levels of Gadd 45 and 153, which are products of stress response genes (19). Because of the putative role of p53 in controlling some apoptotic pathways, we measured the protein levels in MEC lacking MT. The MT null cells had approximately 3–4-fold higher basal levels of p53 than wild-type cells (Fig. 7A). Treatment of MEC with either 3 or 5 μM CDDP for 48 hr resulted in marked increase in p53 protein levels with MT−/− always having higher expression than MT+/+ cells (Fig. 7A). We found no detectable basal levels of Bcl-2 or Bcl-x$_L$ protein levels in either MT+/+ and MT−/− MEC; in
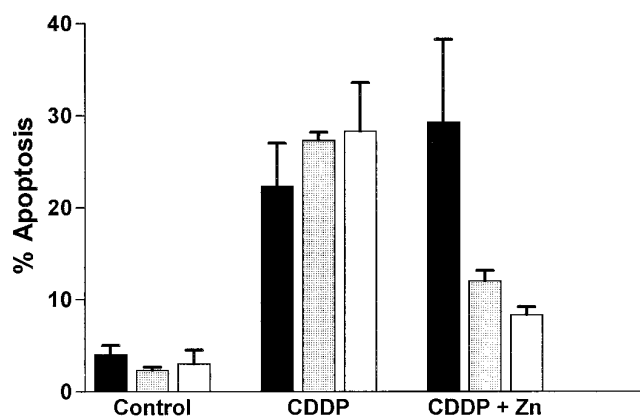
Fig. 6. Apoptosis in primary pulmonary fibroblasts. Pulmonary fibroblasts were pretreated with 0 or 100 μM ZnCl$_2$ for 48 hr, washed three times with drug-free medium, and then treated for 48 hr with 20 μM CDDP. Apoptosis was determined by morphological appearance after staining with (20 μg/ml) Hoescht 33342 as described in Material and Methods. □, MT+/+; ▨, MT+/−; ■, MT−/− (n = 3); *bars,* mean ± standard error.
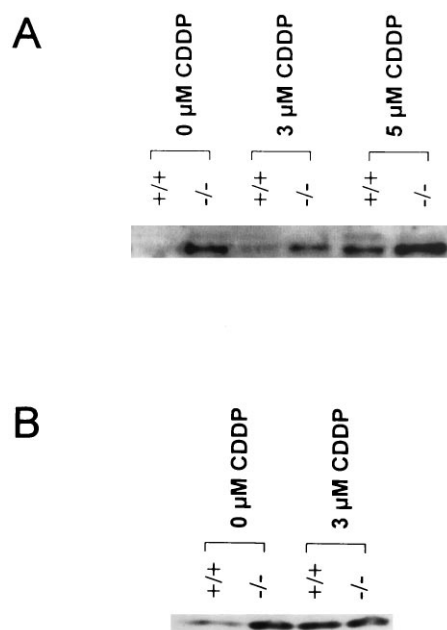
Fig. 7. p53 and Bax protein levels in MEC. A, Immunoblotting of p53 levels in MT−/− and MT+/+ MEC. B, Bax levels in MT−/− and MT+/+ MEC. Cells were treated for 48 hr with 0, 3, or 5 μM CDDP. Protein samples were electroblotted and protein levels determined by Western blotting with anti-p53 and Bax antibodies and chemiluminescent detection. All lanes had similar protein levels as determined by Ponceau S staining.

contrast basal Bax protein levels were higher in MT−/− MEC relative to wild-type cells (Fig. 7B). After treatment with CDDP Bax levels were increased in the wild-type cells to a level approximately equal to that seen in the both untreated and CDDP-treated MT−/− cells (Fig. 7B). We also examined protein extracts from primary pulmonary fibroblasts derived from MT+/+ and MT−/− mice and, consistent with the undetectable MT levels, we found both basal p53 and BAX protein levels similar (data not shown). A 48-hr treatment with 100 μM ZnCl$_2$ did not markedly alter p53 or BAX levels in either primary pulmonary fibroblast populations.

Discussion

Apoptosis is induced by a wide variety of factors including genotoxic anti-cancer drugs, mutagens, oxidants, ionizing irradiation, growth factor withdrawal, survival factor withdrawal, nitric oxide, and nongenotoxic drugs (1–4). There is no compelling evidence for one converging signaling pathway that activates all forms of apoptosis. Nonetheless, several possible mediators of apoptosis have been suggested, including ROI. Despite challenges concerning the role for ROI in apoptosis (11, 12), significant experimental evidence exists supporting this hypothesis. Namely, reduced expression of antioxidant enzymes, such as superoxide dismutase or glutathione peroxidase, increase apoptosis caused by many stimuli (7, 27). Small organic antioxidants, such as Trolox or *N*-acetyl-L-cysteine, protect cells against apoptosis (9, 27). Several apoptotic agonists generate ROI before overt evidence of apoptosis (2, 6). In plants rapid accumulation of H$_2$O$_2$ triggers the hypersensitivity cell death response during infection (28). Oxidants *per se* produce apoptosis in many mammalian model systems. Oncogene products, such as Bcl-2 that protect cells against apoptosis, have been reported to ablate the propagation of some radical species (2, 29, 30). It has been suggested that p53 acts to regulate intracellular redox state and induces apoptosis by a pathway that is dependent on ROI production (9). Redox-sensitive proteins, such as NF-κB, also seem to act in a complex manner to regulate apoptosis (31, 32).

We previously found overexpression of MT produces resistance to electrophilic anti-cancer agents (14), whereas others have observed protection against mutagens (15). Cells that lack MT due to gene deletion are more sensitive to the cytotoxicity of anti-cancer drugs, mutagens, and oxidants (19). Surprisingly, the spectrum of protection provided by MT seems to extend to agents that are not formally electrophilic, including tumor necrosis factor-α and the antimetabolite ara-C. Participation of MT in controlling apoptotic processes, as suggested by our results, could provide an attractive explanation for this protection. Recent studies from other laboratories (33, 34) support a role for MT regulating apoptosis both in all culture and *in vivo*. Mechanistically, the antioxidant properties of MT may be critical. There is now considerable evidence that MT can function as an antioxidant (13, 16, 18, 35–37). Chemical studies have shown that MT is a highly effective reactant with superoxide anion, hydroxyl radical and hypochlorous acid (36). Both MT (35) and Bcl-2 (30) can rescue yeast mutants null for superoxide dismutase from death after oxidant injury. Thus, it seems MT can functionally complement the antiapoptotic protein Bcl-2 in yeast even though its primary amino acid sequence and subcellular localization are not obviously similar to Bcl-2. There is, however, no evidence that MT can complement Bcl-2 in higher eukaryotes and unicellular yeast are not known to undergo formal apoptosis.

It has been reported previously that exogenous zinc mitigates drug-induced internucleosomal DNA damage associated with apoptosis in some cells (12). Although we have not determined the zinc levels in MT null MEC, studies of hepatocytes isolated and cultured from MT null mice did not reveal any significant differences in intracellular zinc pools despite MT being a major intracellular zinc binding protein (38). Furthermore, we found pretreatment with exogenous

zinc (20 μM for 24 hr) did not affect drug-induced apoptosis in MT null MEC (data not shown). This is consistent with a failure of others (26) to identify evidence for internucleosomal DNA damage in MEC by conventional electrophoresis, suggesting a lack of endonuclease activity that zinc might inhibit. Therefore, we believe it is unlikely that the enhanced apoptotic state of MT null cells is a result of altered intracellular zinc levels.

Genotoxins, which cause DNA strand breaks, increase p53 levels. Therefore, we have also tested the hypothesis that increased apoptosis in MT null cell is the result of elevated levels of p53. p53 is a transcription factor that binds to specific DNA sequence elements and activates transcription of a number of gene products, including p21$^{\mathrm{waf\ 1/Cip}}$, Gadd 45, Gadd 153, and Bax (39). Elevated Bax has been associated with enhanced sensitivity to apoptotic agents. *In vitro* experiments have shown p53 is sensitive to oxidation and that the oxidative form of p53 is unable to bind to its cognate DNA *cis*-element (39). Treatment of cells with diethylmaleate, which depletes intracellular pools of the nonprotein thiol glutathione, increases the concentration of oxidative radicals and reduces the ability of p53 to bind its consensus recognition sequences and to activate transcription (39). Diethylmaleate can interact with a wide variety of intracellular protein and non-protein thiols making interpretation of these studies difficult. We found, however, elevated basal p53 levels in our MEC that lack the protein thiol MT but retain similar glutathione levels (18). Results with MCF-7 cancer cells indicate MT antisense can increase p53 (33) and, thus, support our observations. The increased p53 levels in the MT null embryonic cells may result from the modest but reproducible increase in basal DNA damage that was detected by the QFIGE assay (Fig. 4) and the *in situ* assay (Fig. 5), but not by the less sensitive soluble DNA damage assay (Fig. 3). Although we did not examine the DNA binding or transcriptional activity of p53 obtained from MT null cells, the previously reported elevated Gadd 45 and 153 mRNA levels (19) and the increased Bax protein levels observed in this study are consistent with increased p53 transcriptional activity. It is unlikely that all p53 responsive genes are activated, however. Bcl-2 levels were undetectable in MT null and wild-type cells. Furthermore, it seems likely other redox-sensitive proteins may also be involved in the protection afforded by MT against apoptosis as we did not detect markedly reduced p53 and Bax protein levels in primary pulmonary fibroblasts treated with ZnCl$_2$. Interesting candidate proteins include NF-κB (40) and it will be instructive to examine other redox regulated proteins in our model system.

MT seems, therefore, to be a ubiquitous stress-inducible protein that affects the cellular threshold for engagement of apoptosis. Although many apoptotic stimuli such as anticancer drugs can induce MT expression, we believe the kinetics of MT induction are likely to be too slow to permit marked direct protection against the primary stimuli like anticancer drugs. Rather, we would suggest collateral MT induction by endogenous factors, including cytokines, hormones, and drugs, could have a major role in controlling this thresholding process.

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