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Enhanced Sensitivity to Oxidative Stress in Cultured Embryonic Cells from Transgenic Mice Deficient in Metallothionein I and II Genes*

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Embryonic cells from transgenic mice with targeted disruption of metallothionein I and II genes expressed no detectable metallothionein either constitutively or after treatment with cadmium, in contrast to cultured cells that were wild type or heterozygous for the loss of the metallothionein genes. Metallothionein null cells were most sensitive to the cytotoxic effects of cadmium, the membrane permeant oxidant *tert*-butylhydroperoxide, and the redox cycling toxin paraquat. No marked differences were seen among the wild type, heterozygous, or metallothionein null cells in glutathione levels or in the activity of CuZn-superoxide dismutase, glutathione peroxidase, or catalase. Nevertheless, metallothionein null cells were more sensitive to *tert*-butylhydroperoxide-induced oxidation as ascertained by confocal microscopic imaging of dichlorofluoroscein fluorescence. These results indicate basal metallothionein levels can function to regulate intracellular redox status in mammalian cells.

Metallothioneins (MT)[1] are ubiquitous low molecular weight proteins characterized by their unusually high affinity for metals and rich cysteine content. First identified as cadmium-binding proteins (Hamer, 1986), MT are now known to form high affinity complexes with an assortment of trace metals including mercury, platinum, and silver, as well as biologically essential metals like zinc and copper (Hamer, 1986). In many cells MT represents the single most abundant protein thiol source and the major zinc-binding protein.

Basal MT levels can be increased by metals. Treatment of animals or cells with zinc, copper, or cadmium markedly increase MT protein by activation of transcription factors that recognize metal-responsive elements located in the 5′ untranslated region of the MT gene. Although there is general agreement that MT can protect organisms against heavy metal toxicity, it is unlikely that this is the only or even the primary function of MT because these ions are not generally present either endogenously or environmentally in high levels and basal levels of MT are found in most cells. Moreover, gratuitous metal and nonmetal inducers, such as cytokines and drugs, have been identified, which do not bind directly to MT. Thus, MT have been postulated to participate in zinc and copper homeostasis, to regulate the synthesis and activity of zinc metalloproteins, most notably zinc-dependent transcription factors, to protect cells against electrophilic anticancer drugs and mutagens, and to guard against reactive oxygen intermediates (Karin, 1985; Basu and Lazo, 1990).

To test these hypotheses, two general approaches have been used: pharmacologic and genetic. When animals or cells have been treated with pharmacologic or toxicologic inducers of MT, somewhat conflicting conclusions have emerged, perhaps because the agents used have multiple targets (Hamer, 1986; Karin, 1985; Basu and Lazo, 1990). Gene transfer methods also have failed to produce a consensus regarding MT functionality, possibly because of complex and poorly understood MT dose effects or because of additional cellular factors necessary to complement MT.

The widespread phylogenic and cellular expression of MT and its extensive putative functions led most investigators to conclude MT is essential for development. Thus, the recent successful production of MT null mice was surprising (Michalska and Choo, 1993; Masters *et al.*, 1994). In initial studies, these mice reproduced normally and displayed few overt abnormalities other than increased sensitivity to cadmium toxicity. We now report on the phenotypic characteristics of embryonic cells isolated from these mice.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—Primary mouse embryonic cells (MEC) were prepared according to the method of Todaro and Green (1963) as modified by Harvey *et al.* (1993). Heterozygous mice (MT I and II +/−; Michalska and Choo, 1993) of 129 Ola and C57Bl/6 mixed genetic background were mated and fetuses isolated from 14.5-day pregnant females. Individual embryos were homogenized by passing through a 1-cm^3 syringe and 19-gauge needle and the cell suspension plated on gelatinized 10-cm Petri dishes. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cell were genotyped by polymerase chain reaction as described by Michalska and Choo (1993). Because MT −/− cells appeared to proliferate better in 20% serum, after passage 4 and for all studies in this report, we have cultured all cells in the above mentioned medium and 20% fetal bovine serum. Cells were grown at 37°C in 5% CO_2, passaged every 3 days and routinely found free of mycoplasma. Unless noted otherwise, all chemicals were obtained from Sigma.

MT and Antioxidant Determinations—Cell lysates were prepared by repetitive freezing and thawing in Tris buffer (10 mM) containing $CdCl_2$ (10 μM). After ultrasonic disruption, the supernatant fraction was analyzed for protein content using a modified Bradford technique and MT

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[1] The abbreviations used are: MT, metallothionein(s); MEC, mouse embryonic cell(s); tBH, *tert*-butylhydroperoxide; HBSS, HEPES-buffered salt solution; DCF, dichlorofluoroscein; DCFH, dichlorofluoroscin; DCFHDA, dichlorodihydrofluorescein diacetate.

was measured by a modification of our ^{109}Cd binding assay (Schwarz *et al.*, 1994). Values less than twice background (approximately 5 ng/mg protein) were considered undetectable. Total glutathione (GSH) content in sonicated cell homogenates was determined by the GSH reductase 5,5'-dithiobis(2-nitrobenzoic acid) assay of Tietze (1967). Catalase and CuZn-superoxide activities were measured by the method of Wheeler *et al.* (1990). Enzymatic glutathione peroxidase activity in both sonicated cell homogenates and high molecular mass protein fractions (>10,000 Da) produced by centrifugation through a Microcon 10 filter (Amicon, Inc., Beverly, MA) was measured by the standard indirect method based on NADPH oxidation *tert*-butylhydroperoxide (tBH) in the presence of glutathione and glutathione reductase (Günzler and Flohé, 1985). For MT immunolocalization, MEC growing on eight-well chamber microscopic slides were examined by confocal microscopy with a previously described MT antiserum (M2) directly linked to the fluorescent probe FluoroLink Ab Cy3 (Biological Detection Systems, Pittsburgh, PA) (Kuo *et al.*, 1994).

Cytotoxicity—Cell sensitivity to the cytotoxic effects of $CdCl_2$, tBH, and paraquat was determined using our previously described microtiter assay (Schwarz *et al.*, 1994). Briefly, single cell suspensions were obtained after treatment of adherent cultures with 0.1% trypsin, 0.4 mM EDTA in Puck's Saline A for 3 min. Cells were resuspended in medium with fetal bovine serum and plated at 2,000 cells/well in 96-well plates. After a 24-h incubation at 37 °C, cells were exposed for 1 h to $CdCl_2$ (0–1 mM), tBH (0–3 mM), or paraquat (0–2 mM) in serum-free Dulbecco's modified Eagle's medium. The cadmium- or oxidant-containing medium was then removed, the cells washed twice with serum-containing medium, and fresh cadmium/oxidant-free, serum-containing medium added. After incubation in humidified 37 °C incubator (5% CO_2) for 4–6 days, the viability of subconfluent cells (4–6 replicates/concentration) was quantified using reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and a multiplate reader (Titertek Multiscan Plus) at 540 nm (Schwarz *et al.*, 1994). The results from two to four separate experiments with 8–16 determinations were used to estimate cellular sensitivity to $CdCl_2$, tBH, or paraquat. For some morphological studies, cells were exposed to $CdCl_2$ for 24 h.

Determination of Intracellular Oxidants—The fluorescent probe dichlorodihydrofluorescein diacetate (DCFHDA; Molecular Probes, Eugene, OR) was used with confocal microscopy to monitor net intracellular generation of reactive oxygen species in the absence or presence of tBH (Schwarz *et al.*, 1994). MEC, growing on 31-mm glass coverslips, were rinsed in HEPES-buffered salt solution (HBSS) and loaded with 10 μM DCFHDA dye in HBSS supplemented with 5 mg/ml bovine serum albumin for 15 min at 37°C. Cells were rinsed with HBSS and mounted in a recording chamber. Recordings were made at room temperature using a Meridian Instruments ACAS 570c Imaging System in the confocal mode. Cells were illuminated with an argon laser (488 nm; 200 milliwatts). The laser was set at 200 milliwatts and illuminating light attenuated by passage through an acousto-optical modulator that eliminated 95% of the signal and by passage through a 7% neutral density filter. Intracellular antioxidant activity was assessed by monitoring fluorescence due to oxidation of dichlorofluoroscin (DCFH) to dichlorofluoroscein (DCF). Images were obtained from a minimum of 15 individual cells. After mounting the glass coverslips and adding HBSS, two determinations were made to assess basal autoxidation. Following aspiration of the HBSS recording chamber solution, a fresh solution of HBSS and 500 μM tBH was added. This concentration has been found previously to produce a detectable response in mammalian cells (Schwarz *et al.*, 1994). Eight additional images were then obtained, 1 min apart. Fluorescence intensity based on average pixel intensity in delimited cellular areas was measured as described previously with the Meridian Instruments ACAS software (Schwarz *et al.*, 1994).

Statistics—Data were analyzed by Student's *t* test or by ANOVA with Bonferroni correction for multiple comparisons.

RESULTS

Biochemical and Morphological Characteristics—Basal MT levels were 0.11 ± 0.04 and 0.04 ± 0.01 μg/mg protein in wild type and heterozygous MEC, respectively, with the approximate 2-fold difference in MT levels in agreement with gene dosage. MT −/− cells had no detectable basal MT (Fig. 1). Both wild type and heterozygous cells displayed a cadmium concentration-dependent increase in MT. In contrast, MT levels remained below the limits of detection in MT −/− cells exposed to cadmium (Fig. 1). Fluorescence immunolocalization studies confirmed the absence of MT in the MT −/− cells and revealed a nuclear distribution in the wild type cells (data not shown).

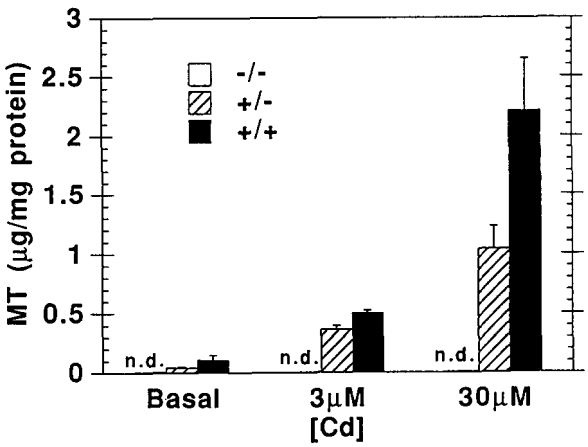

FIG. 1. **MT levels in MEC.** MT was measured by radiolabeled cadmium binding in heat-stable low molecular protein extracts from MEC. The mean results of three or more determinations are shown. *Bars* equal S.E. *Hatched symbols* are MT +/−, and *black symbols* are MT +/+. n.d. = not detectable.

The morphology of the three MEC were similar when the cells were cultured with 20% fetal bovine serum in complete medium (Fig. 2). A 24-h exposure to 30 μM cadmium was associated with considerable cytotoxicity in MT −/− but not MT +/+ cells; MT +/− cells had an intermediate response to cadmium (Fig. 2). Deletion of MT I and MT II genes did not significantly alter levels of the major nonprotein thiol GSH nor the activity of the antioxidant enzymes CuZn-superoxide dismutase, glutathione peroxidase, or catalase in total cell homogenates (Table I). To exclude potential interference in glutathione peroxidase assay from endogenous MT in wild type and heterozygous cells, we also examined high molecular mass homogenates (>10 kDa) and found no significant differences in the glutathione peroxidase activities (data not shown).

Cellular Sensitivity to Cadmium, tert-Butylhydroperoxide, and Paraquat—Wild type MEC were less sensitive to the growth inhibitory effects of a 1-h cadmium exposure than either heterozygous or MT null cells (Fig. 3*A*). The cadmium concentrations required for 50% growth inhibition in MT +/+, +/−, and −/− cells were 250, 150, and 70 μM, respectively. Loss of basal MT expression also increased cell sensitivity to tBH (Fig. 3*B*). MT −/− cells were more sensitive to tBH compared to MT +/+ cells at tBH concentrations > 3 μM, and approximately 2–3-fold more tBH was required to produce an equivalent inhibition in cell growth in MT +/+ than −/− cells. MT +/− cells were as resistant as wild type cells at tBH concentrations up to 30 μM; at >30 μM tBH the heterozygous cells were as sensitive as the MT null cells consistent with a threshold requirement for MT. A steep concentration response to the dioxygen-dependent redox cycling herbicide paraquat was noted with all three cell types (Fig. 3*C*). Nevertheless, the MT −/− cells were also significantly more sensitive to paraquat than MT +/+ cells at all concentrations > 100 μM ($p < 0.05$) and approximately 2-fold less paraquat was required to produce 50% growth inhibition. As with tBH the partial loss of basal MT expression in the heterozygous cells resulted in increased sensitivity to paraquat compared to the wild type cells, although no threshold was apparent.

The sensitivity of null cells to tBH was examined further using the oxidant-responsive fluorescent probe DCFHDA. Cells were preloaded with 10 μM DCFHDA for 15 min and then treated briefly with 500 μM tBH. The conversion of DCFH to DCF was monitored by the appearance of a fluorescent signal in live cells using confocal microscopy and provided relative

quantitative information regarding the steady-state oxidant burden. The basal level of DCF formation in MT −/− cells was not significantly greater than in either heterozygous or wild type cells. Within 1 min after tBH, additional marked intracellular oxidation of DCFH occurred (Figs. 4 and 5). In MT null cells, a 10-fold increase in fluorescence over the initial fluorescence was sustained for at least 8 min after tBH treatment (Fig. 5). A 8- and 5-fold increase, in heterozygous and wild type MEC, respectively, was observed during the tBH treatment (Fig. 5). At all times the most intense fluorescent signal in MT −/− cells was localized centrally in an area corresponding to the nucleus determined by simultaneous phase microscopy (Fig. 4).

DISCUSSION

The ubiquitous cellular and phylogenic distribution of MT implies important physiological functions. These putative functions remain unclear in spite of a wealth of structural and molecular biological information regarding MT. Nonetheless, a role for MT in detoxifying heavy metals is apparent (Hamer, 1986; Karin, 1985), and support for a protective role of MT against reactive oxygen species is accumulating (Thornalley and Vasak, 1985; Sato and Bremner, 1993; Chubatsu and Meneghini, 1993; Tamai *et al.*, 1993). MT is thought to have a detoxifying role against metals such as cadmium because: (*a*) MT transcription is induced by cadmium (Karin, 1985); (*b*) MT forms high affinity thiolate clusters with cadmium, which reduces the ability of the metal to react with other biomolecules (Kagi and Schaffer, 1988); (*c*) cultured cells selected for resistance to cadmium have elevated MT levels (Hamer, 1986; Basu and Lazo, 1990; Kelley *et al.*, 1988); and (*d*) overexpression of MT after gene transfer reduces the sensitivity of a variety of cells to cadmium (Basu and Lazo, 1990; Kelley *et al.*, 1988; Kaina *et al.*, 1990; Koropatnick and Pearson, 1993). The most compelling support, however, is derived from two independent studies in which targeted disruption of MT I and MT II genes in mice enhances their sensitivity to cadmium (Michalska and Choo, 1993; Masters *et al.*, 1994). In the current report, we demonstrate that embryonic cells isolated from a transgenic mouse deficient in MT I and MT II genes retain enhanced sensitivity to cadmium (Figs. 2 and 3).

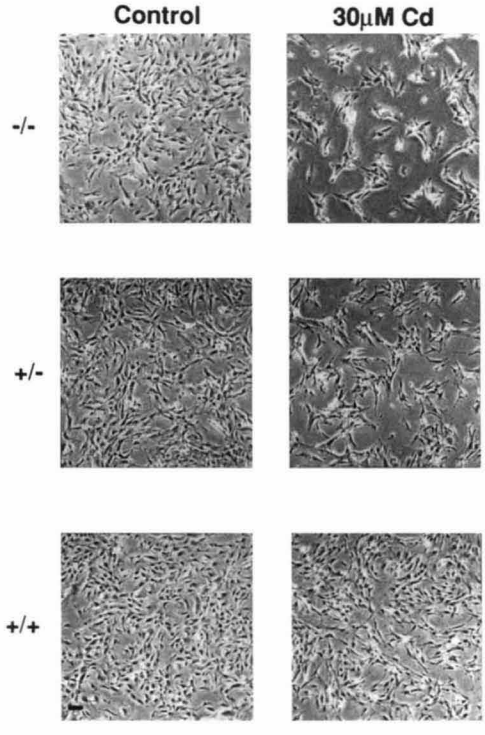

FIG. 2. **Morphology of wild type, heterozygous and MT null cells after treatment with cadmium.** MT −/−, +/−, and +/+ cells were treated with vehicle or 30 μM $CdCl_2$ for 24 h. *Bar* = 50 μm.

In contrast to the involvement of MT in heavy metal detoxification, support for a role for MT as an antioxidant is more circumstantial, ambiguous, and controversial. Nonetheless, there are several reasons for believing MT could function as an antioxidant in mammalian cells. First, a variety of physical and chemical stresses known to be associated with oxidative injury increase MT gene expression. X-irradiation (Koropatnick *et al.*, 1989), hyperoxia (Veness-Meehan *et al.*, 1991), and restraint (Bauman *et al.*, 1991) result in tissue-specific increases in oxygen free radicals and mRNA for MT expression. Xenobiotics, such as paraquat (Bauman *et al.*, 1991) and CCl_4 (Hidalgo *et al.*, 1988), and anticancer drugs, such as doxorubicin, cisplatin and bleomycin (Basu and Lazo, 1990; Bauman *et al.* 1991), have been reported to have similar effects *in vivo*. These stimuli, however, can cause an inflammatory response characterized by the production of cytokines, such as interleukin-1, interleukin-6, tumor necrosis factor α, and interferon γ, which can increase MT gene expression (Hamer, 1986; Karin, 1985; Schroeder and Cousins, 1990; De *et al.*, 1990). Thus it is unresolved if reactive oxygen radicals *per se* or secondary cytokines affect MT expression *in vivo*. It is intriguing, however, that MT expression has recently been found to be responsive to oxygen tension in cultured tumor cells (Murphy *et al.*, 1994). Second, MT induction with heavy metals or cytokines reduces cell sensitivity to oxidant injury caused by ionizing radiation (Basu and Lazo, 1990), hydrogen and organic peroxides (Schwarz *et al.*, 1994; Mello-Filho *et al.*, 1988), hyperoxia (Hart *et al.*, 1990), CCl_4 (Schroeder and Cousins, 1990), and tumor necrosis factor (Leyshon-Sorland *et al.*, 1993). Heavy metals and cytokines are promiscuous transcriptional activators, however, and cadmium or zinc pretreatment can produce an H_2O_2-resistant cell as a result of activating non-MT thiol antioxidants (Chubatsu *et al.*, 1992). Third, overexpression of MT via direct gene transfer has been shown to reduce the sensitivity of lower (Tamai *et al.*, 1993) and higher (Schwarz *et al.*, 1994) eukaryotes to oxidative injury. Other investigators, however, have failed to detect altered resistance to oxidative stress in Chinese hamster ovary cells that overexpress human MT IIA after gene transfer (Kaina *et al.*, 1990), suggesting the protection offered by MT to oxidant injury may be complex. Fourth, underexpression of MT with antisense oligonucleotides restores the H_2O_2 sensitivity of

TABLE I

Superoxide dismutase, catalase, and glutathione peroxidase activities and glutathione content of MEC

Each value is the mean ± S.E. from three to four independent determinations. All activities were determined in cell homogenates as described under "Experimental Procedures."

MT MEC	Superoxide dismutase	Catalase	Glutathione	Glutathione peroxidase
	units/mg protein	*units/mg protein*	*nmol/mg protein*	*units/mg protein*
−/−	2.63 ± 0.04	0.62 ± 0.13	15.6 ± 2.2	169 ± 27
+/−	2.63 ± 0.04	0.72 ± 0.10	13.8 ± 1.0	235 ± 30
+/+	2.64 ± 0.05	0.70 ± 0.09	16.6 ± 2.9	178 ± 8

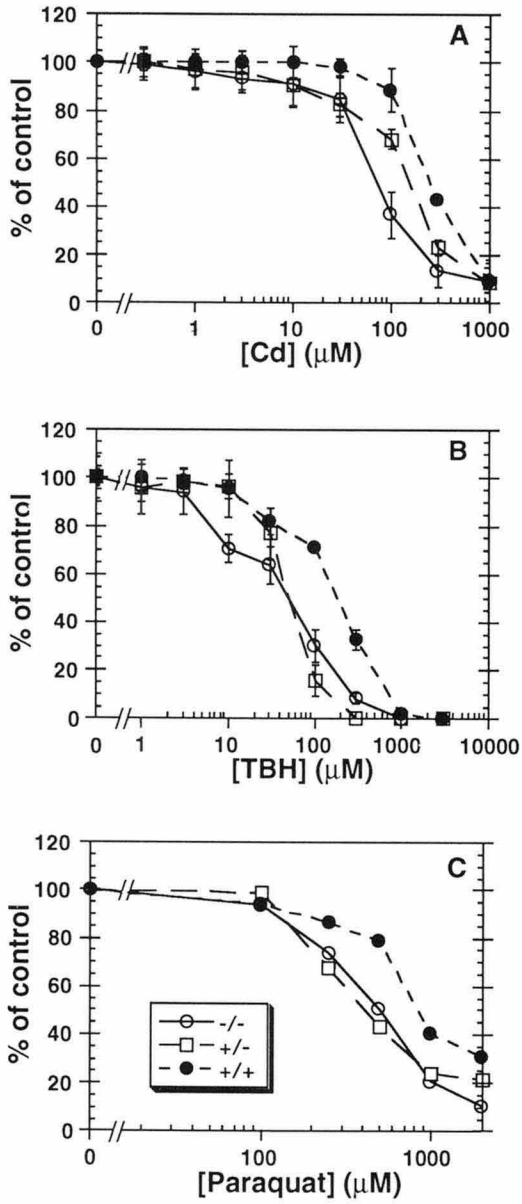

FIG. 3. **Survival of MEC after $CdCl_2$, tBH, or paraquat treatment.** Cells were incubated with $CdCl_2$ (*panel A*), tBH (*panel B*), or paraquat (*panel C*) for 1 h, and cell survival was measured 4–6 days later by a colorimetric assay. The symbols represent the mean values from 8 (*panels A* and *B*) to 16 (*panel C*) determinations. *Bars* = S.E., unless smaller than the symbol. *Open circles*, −/−; *open squares*, +/−; *closed circles* +/+.

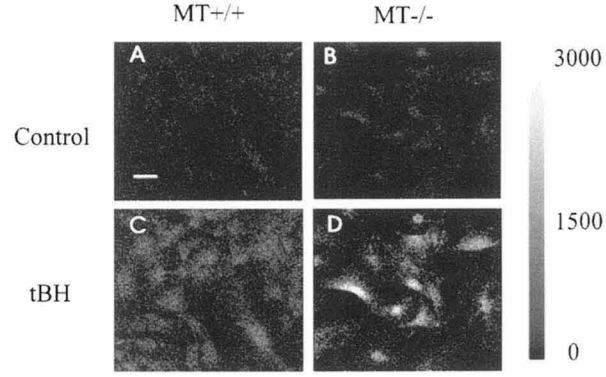

FIG. 4. **Formation of dichlorofluoroscein in MEC.** Cells were exposed to 500 μM tBH and the intracellular fluorescence determined 480 s later microscopically. *A*, MT +/+ untreated; *B*, MT −/− untreated; *C*, MT +/+ treated with 500 μM tBH; *D*, MT −/− treated with 500 μM tBH. *Bar* = 30 μm.

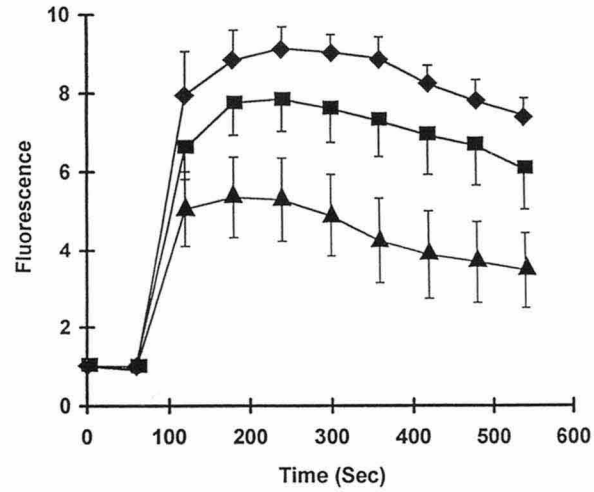

FIG. 5. **Kinetics of dichlorofluoroscein formation in MEC.** Representative results from four independent experiments were normalized to initial fluorescent values. The mean fluorescence values obtained from 15–20 cells are shown with S.E. tBH was added after the second determination at 60 s. *Circles*, MT −/−; *triangles*, MT +/−; *squares*, MT +/+.

Chinese hamster lung fibroblasts with previous elevated nuclear MT content (Chubatsu and Meneghini, 1993).

MT null mouse cells provide a more direct and simple model with which to examine the role of MT as a member of the intracellular antioxidant network. The ability to totally deplete cells of MT I and MT II protein has not been achieved with antisense techniques (Leibbrandt *et al.* 1994). Moreover, we were unable to express MT protein even with a strong stimulus such as cadmium (Fig. 1), in contrast to either the heterozygous or wild type cells. Thus, the MT null cells present a useful model to dissect the effects of heavy metals in the absence of MT induction. Finally, the MT −/− cells were similar to the control cells with respect to activity of several important antioxidants (Table I).

In a previous report (Schwarz *et al.*, 1994), we noted MT overexpression after plasmid transfection reduced the sensitivity of NIH3T3 cells to tBH, a membrane permeant oxidant thought to kill mammalian cells by peroxidizing membrane lipids (Masaki *et al.*, 1989). tBH-mediated DNA damage was not reduced in cells overexpressing MT (Schwarz *et al.*, 1994). This oxidant can produce a complex variety of reactive oxygen intermediates within cells (Royall and Ischiropoulos, 1993; LeBel *et al.*, 1992). In the current report, the MT −/− cells demonstrated an enhanced sensitivity not only to tBH but also the redox cycling herbicide paraquat, which is structurally quite distinct from tBH. Although the magnitude of the protection against tBH and paraquat afforded by the presence of both functional MT alleles in the wild type cells may seem small (approximately 2–3-fold) compared to the MT null cells, it is comparable to the protective effect against tBH when MT is overexpressed 4-fold in NIH3T3 cells (Schwarz *et al.*, 1994) or against paraquat when catalase is overexpressed 100-fold in L cells (Speranza *et al.*, 1993) by gene transfer. Therefore, we believe these changes in sensitivity could have biological significance.

The lack of MT resulted in an impaired ability of the MT null cells to quench the tBH-induced change in fluorescence of the oxidant-sensitive dye, DCFH. The relative stability of the fluorescent signal after tBH addition illustrates the advantage of live cell confocal microscopic methods to detect DCFH oxida-

tion. We saw little evidence for cellular loss of oxidized DCFH when cells were attached to coverslips in contrast to previously described studies with detached cells (Royall and Ischiropoulos, 1993). Although DCFH is a valuable probe to identify the generation of intracellular reactive oxygen species, the precise species responsible for the oxidation of DCFH to DCF are not known (LeBel *et al.*, 1992). It appears unlikely that superoxide anion or H_2O_2 alone directly oxidize DCFH; H_2O_2-Fe^{2+}-derived oxidants may be primarily responsible for oxidation of DCFH at least based on *in vitro* results (LeBel *et al.*, 1992). The mechanism by which MT acts as an antioxidant is unclear, although it can scavenge phenoxyl radicals *in vitro* as determined by electron spin resonance (Schwarz *et al.*, 1994). Previous reports indicated that partially purified MT is capable of scavenging hydroxyl and superoxide anions *in vitro* (Thornalley and Vasak, 1985; Thomas *et al.*, 1986; Hainaut and Milner, 1993). Thus, the cysteine residues of MT might serve as an expendable target for reactive oxygen species, but such a mechanism does not explain the failure to see protection against oxidants after MT overexpression in some studies (Kaina *et al.*, 1990). Alternatively, the antioxidant properties of MT may rely on its metal speciation and the ability of oxygen free radicals to release zinc from MT thiolate clusters and the antioxidant activity of zinc on plasma membranes and on other nuclear and cytoplasmic proteins (Thomas *et al.*, 1986; Hainaut and Milner, 1993). It is particularly interesting that the DCF signal appeared to be localized in a discrete area intracellularly, most probably corresponding to the nucleus, and that MT colocalized to this area in the heterozygous and wild type cells. Clearly more attention should be directed to the functional significance of nuclear MT.

MT is expressed constitutively at low levels in virtually all cell types. The MT null cells provide a convenient tool to explore various roles of MT without elevating MT levels, either pharmacologically or genetically, to levels that often exceed normal physiological values. Our results indicated loss of MT yielded cells that were more sensitive to tBH and paraquat. Since other antioxidant defense mechanisms appeared not to compensate in the MT null cells, a singularly important value for constitutive levels of MT is apparent. The antioxidant role of MT could suggest the MT null mice may have altered sensitivity to pro-oxidant and inflammatory pathophysiologic states.

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REFERENCES

Basu, A., and Lazo, J. S. (1990) *Toxicol. Lett.* **50,** 123–135
Bauman, J. W., Liu, L., Liu, Y. P., and Klaassen, C. D. (1991) *Toxicol. Appl. Pharmacol.* **110,** 347–354
Chubatsu, L. S., and Meneghini, R. (1993) *Biochem. J.* **291,** 193–198
Chubatsu, L. S., Gennari, M., and Meneghini, R. (1992) *Chem. Biol. Interact.* **82,** 99–110
De, S. K., McMaster, M. T., and Andrews, G. K. (1990) *J. Biol. Chem.* **265,** 15267–15274
Günzler, W. A., and Flohé, L. (1985) in *CRC Handbook of Methods for Oxygen Radical Research,* 3rd Ed., pp. 285–290, (Greenwald, R. A., ed) CRC Press, Inc., Boca Raton, FL
Hainaut, P., and Milner, J. (1993) *Cancer Res.* **53,** 4469–4473
Hamer, D. H. (1986) *Annu. Rev. Biochem.* **55,** 913–952
Hart, B. A., Voss, G. W., Shatos, M. A., and Doherty, J. (1990) *Toxicol. Appl. Pharmacol.* **103,** 255–270, 1990
Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., Giovanella, B. C., Tainsky, M. A., Bradley, A., and Donehower, L. A. (1993) *Oncogene* **8,** 2457–2467
Hidalgo, J., Campany, L., Borras, M., Garvey, J. S., Armario, A. (1988) *Am. J. Physiol.* **255,** E518–E524
Kagi, J. H. R., and Schaffer, A. (1988) *Biochemistry* **27,** 8509–8515
Kaina, B., Lohrer, H., Karin, M., and Herrlich, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2710–2714
Karin, M. (1985) *Cell* **41,** 9–10
Kelley, S. L., Basu, A., Teicher, B. A., Hamer, D. H., Hacker, M. P., and Lazo, J. S. (1988) *Science* **241,** 1813–1815
Koropatnick, J., and Pearson, J. (1993) *Mol. Pharmacol.* **44,** 44–50
Koropatnick, J., Leibbrandt, M., and Cherian, M. G. (1989) *Radiat. Res.* **119,** 356–365
Kuo, S.-M., Kondo, Y., DeFilippo, J. M., Ernstoff, M. S., Bahnson, R. R., and Lazo, J. S. (1994) *Toxicol. Appl. Pharmacol.* **125,** 104–110
LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.* **5,** 227–231
Leibbrandt, M. E. I., Khokha, R., and Koropatnick, J. (1994) *Cell Growth & Diff.* **5,** 17–25
Leyshon-Sorland, K., Morkrid, L., and Rugstad, H. E. (1993) *Cancer Res.* **53,** 4874–4880
Masaki, N., Kyle, M. E., and Farber, J. L. (1989) *Arch. Biochem. Biophys.* **269,** 390–399
Masters, B. A., Kelly, E. J., Quaife, C. J., Brinster, R. L., and Palmiter, R. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 584–588
Mello-Filho, A. C., Chubatsu, L. S., and Meneghini, R. (1988) *Biochem. J.* **256,** 475–479
Michalska, A., and Choo, K. H. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8088–8092
Murphy, B. J., Laderoute, K. R., Chin, R. J., and Sutherland, R. M. (1994) *Cancer Res.* **54,** 5808–5810
Royall, J. A., and Ischiropoulos, H. (1993) *Arch. Biochem. Biophys.* **302,** 348–355
Sato, M., and Bremner, I. (1993) *Free Radical Biol. Med.* **14,** 325–337
Schroeder, J. J., and Cousins, R. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3137–3141
Schwarz, M., Lazo, J. S., Yalowich, J. C., Reynolds, I., Kagan, V. E., Tyurin, V., Kim, Y.-M., Watkins, S. C., and Pitt, B. R. (1994) *J. Biol. Chem.* **269,** 15238–15243
Speranza, M. J., Bagley, A. C., and Lynch, R. E. (1993) *J. Biol. Chem.* **268,** 19039–19043
Tamai, K. T., Gralla, E. B., Ellerby, L. M., Valentine, J. S., and Thiele, D. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8013–8019
Thomas, J. P., Bachowski, G. J., and Girotti, A. W. (1986) *Biochim. Biophys. Acta* **884,** 448–461
Thornalley, P. J., and Vasak, M. (1985) *Biochim. Biophys. Acta* **827,** 36–44
Tietze, F. (1969) *Anal. Biochem.* **27,** 502–522
Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17,** 299–313
Veness-Meehan, K. A., Cheng, E. R. Y., Mercier, C. E., Blixt, S. L., Johnston, C. J., Watkins, R. H., and Horowitz, S. (1991) *Am. J. Respir. Cell Mol. Biol.* **5,** 516–521
Wheeler, C. R., Salzman, J. A., Elsayed, N. M., Omaye, S. T., and Korte, Jr., D. W. (1990) *Anal. Biochem.* **184,** 193–199