Cytoplasmic Metallothionein Overexpression Protects NIH 3T3 Cells from tert-Butyl Hydroperoxide Toxicity*

(Received for publication, January 25, 1994)

Margaret A. Schwarz‡‡, John S. Lazo†, Jack C. Yalowich§, Ian Reynolds¶¶, Valerian E. Kagan**, Vladimir Tyurin***§§, Young-Meong Kim‡‡‡, Simon C. Watkins†††, and Bruce R. Pitts‡

From the Departments of Pharmacology, Anesthesiology and Critical Care Medicine, **Environmental and Occupational Health, §§Surgery, and ¶¶Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Metallothioneins (MT) are ubiquitous low molecular weight metal-binding proteins that may act as antioxidants. We examined the sensitivity of NIH 3T3 cells transfected with a plasmid containing mouse metallothionein-I gene (NIH3T3/MT) to the membrane permeant oxidant, tert-butyl hydroperoxide (tBH). NIH3T3/MT cells had a 4-fold increase in intracellular metallothionein as compared to cells transfected with a plasmid containing an inverted gene (NIH3T3/TM). Newly expressed metallothionein appeared to be localized to the cytoplasm as determined by immunofluorescence and confocal microscopy. NIH3T3/MT cells were 6 times more resistant than NIH3T3/TM cells to the cytotoxic effects of tBH. The antioxidant activity of NIH3T3/MT cells was greater than NIH3T3/TM cells, since exposure to tBH resulted in significantly less (a) thiobarbituric acid-reactive substances and (b) fluorescence after loading cells with the oxidant-sensitive dye, 2'7'-dichlorodihydrofluorescein diacetate. Furthermore, homogenates of NIH3T3/MT cells were more capable of scavenging in vitro generated phenoxyl radicals as quantified by electron spin resonance detection. In contrast, overexpression of cytoplasmic MT did not protect against tBH-induced DNA damage, suggesting that subcellular location of MT is important for its function and that DNA damage is not a key determinant of cytotoxicity. These data provide direct support for an antioxidant role for MT, since physiologically relevant elevations in cytoplasmic MT interfere with tBH-induced cytotoxic peroxidation.

Metallothioneins (MT) are low molecular mass (<7 kDa), cysteine-rich (30 mol %) proteins for which the biological functions remain ill defined (1). One likely role for MT involves intracellular metal ion homeostasis, since transcription of MT is induced by heavy metals such as zinc, cadmium, and copper; MT binds these same heavy metals (2). MT may also participate in defense against intracellular oxidants. Support for this possibility is derived from observations that: (a) MT genes are transcriptionally activated in cells (3) and tissues (4) during oxidative stress, and (b) in vitro, MT can scavenge free hydroxyl radicals and superoxide anions produced by xanthine-xanthine oxidase reaction (5), as well as inhibit degradation of isolated DNA by hydroxyl radicals (6). Although these latter (5, 6) observations are supportive of an antioxidant function for MT, its role as an antioxidant in intact cells remains unclear. Overexpression of MT with heavy metals or cytokines produces resistance to oxidant injury caused by ionizing radiation (7), hydrogen (8) and organic peroxides (9), hypoxia (10), and carbon tetrachloride (11). Heavy metals and cytokines are promiscuous transcriptional activators, however, and may affect other cellular proteins in addition to MT. For instance, it appears that glutathione and not MT accounts for resistance of yeast or simian metallothionein proteins, in the presence of copper, suppressed oxidant injury in Saccharomyces cerevisiae mutants lacking copper-zinc superoxide dismutase (13). The protection afforded these mutants by MT was limited to injury caused by the accumulation of free radicals when the yeast were grown on the non-fermentable carbon source, lactate, and it was reported to not affect their sensitivity to high oxygen or paraquat. Furthermore, the antioxidant effect of MT appeared limited to the Cu(I)-speciated form of MT. Thus, we examined the potential antioxidant properties of MT by elevating cytoplasmic MT to a physiologically level via gene transfer in higher eukaryotes that have functional CuZn-superoxide dismutase.

We transferred the mouse MT-I gene into NIH 3T3 cells and noted a 4-fold enhancement of MT localized to the cytoplasm. The resulting NIH 3T3 cells were almost 10-fold more resistant than control cells to the cytotoxic effects of tert-butyl hydroperoxide (tBH), a membrane permeant oxidant thought to kill mammalian cells by peroxidizing membrane lipids (14). Cells that overexpressed MT had a greater antioxidant activity than control cells as revealed by: (a) accumulation of less intracellular peroxidation products upon exposure to tBH (as determined using the oxidant-sensitive fluorophore dichlorodihydrofluorescein diacetate and thiobarbituric acid-reactive substances), and (b) greater ability to scavange phenoxyl radical (as determined by electron spin resonance (ESR)).

EXPERIMENTAL PROCEDURES

Cell Culture—NIH 3T3 cells were purchased from American Type Culture Collection (CRT 1658) and maintained in 10% fetal bovine serum (Hyclone, Logan, UT), penicillin and streptomycin, HEPES buffer, and Dulbecco's modified Eagle's medium. Cells were grown at 37 °C in 5% CO₂ and were routinely found free of mycoplasma.

Transfection—Plasmids pBPVGRPMT and pBPVGRPTM were generous gifts from Dr. Kathryn A. Morton (University of Utah, Salt Lake City) and were defrayed in part by the payment of page charges. This article must be typed in accordance with the specifications of the American Society for Biochemistry and Molecular Biology, Inc. © 1994 by The American Society for Biochemistry and Molecular Biology, Inc.
City, UT). Both plasmids contain the genome of bovine papilloma virus (BPV) that is useful for maintaining multiple copies of the plasmid for transcription (15). In pBPVGRPMT, mouse metallothionein-I gene was inserted in sequence and separated from its GRP promoter. Cells transfected with this plasmid (BPV) that is useful for maintaining multiple copies of the plasmid for repetitive freezing and thawing in Tris buffer (10 mM) containing 50% glycerol, were then studied over the ensuing 1-6 weeks, during which time intracellular MT was quantified routinely (see below). In addition, we also studied cells transfected with pSV2neo alone (NIH3T3/Neo) and selected in G-418 or native NIH 3T3 cells (control).

Cellular Metallothionein Determination—Cell lysates were prepared by repetitive freezing and thawing in Tris buffer (10 mM) containing 50% glycerol, were then studied over the ensuing 1-6 weeks, during which time intracellular MT was quantified routinely (see below). In addition, we also studied cells transfected with pSV2neo alone (NIH3T3/Neo) and selected in G-418 or native NIH 3T3 cells (control).

Cytotoxicity—Cells (5000) were transferred to 96-well Lab-Tek chamber slides (Nunc, Naperville, IL). At middle logarithmic growth phase, cells were rinsed twice with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS. After being permeabilized for 15 min with 0.1% Triton X-100 in 2% paraformaldehyde and phosphate-buffered saline, cells were rinsed with PBS and nonspecificity of antibody blocked with a 0.5% bovine serum albumin, 0.15% glycine, and 5% goat serum solution (buffer A) for 30 min. Cells were then fixed at room temperature for 1 h with buffer A, a solution containing a 1:100 dilution of a previously described (18) affinity-purified rabbit antisemur that recognizes all major MT isoforms. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min. Chambers were then rinsed with buffer A and with Cy3 Fluorolink-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) for 40 min.

DNA Single-strand Breaks—tBH-induced DNA damage was assessed using a modified alkaline elution technique for high frequency single-strand breaks (21). NIH3T3/MT and TM cells were labeled with [2,4-3H] hypoxanthine and then exposed to tBH (6.6 lCi/ml). NIH 3T3 cells incubated with [3H]thymidine for 48 h, and containing [3H]thymidine, received 15 Gy irradiation from a 100 Ci source irradiator (Gamma cell 1000 Mark Irradiator; J. L. Sheppard and Associates, Glendale, CA; 4.15 Gy/min) and were added as internal standards. Cells were labeled on polyvinyl chloride filter (0.8 µm; Gelman Sciences, Inc, Ann Arbor, MI) and lysed with 2% sodium dodecyl sulfate, 10 mmoiidisodium EDTA, and 0.9 mg/ml proteinase K at pH 10.0. DNA was eluted from the filter after addition of tetrapropylammonium hydroxide (50 µl) and heated at 50°C for 10 min. DNA was desalted in the column using standard liquid scintillation spectrometry and the frequency of tBH-induced DNA strand breakage was quantified as the fraction of [3H]DNA remaining on the filter when 75% of internal [3H]DNA remained. A calibration curve for relating frequency of tBH-induced DNA strand breaks to corresponding effect of ionizing radiation using [3H]labeled cells was obtained by plotting radiation exposure (Gy) versus [3H]DNA retention of the [3H]DNA internal standard.

Determination of Intracellular Oxidants and Lipid Peroxidation Products—The fluorescent probe dichlorodihydrofluorescein (H2DCF; Molecular Probes, Eugene, OR) was used to monitor net intracellular generation of reactive oxygen species by tBH (22). NIH3T3/MT and TM cells were grown on 31-mm glass coverslips. Cells were rinsed in HEPES-buffered salt solution (HBSS) and loaded with 10 µM H2DCF diacetate dye in HBSS supplemented with 5 mM 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 an ACAS 570c imaging system (Meridian Instruments, East Lansing, MI) in the confocal mode. Cells were illuminated with an argon laser (488 nm; 200 mW). The laser was set at 200 mW and illuminating light attenuated by passage through an acousto-optical modulator that eliminated 95% of 488 nm light and also by passing the image through a 520 nm long pass filter. Intracellular antioxidant activity was assessed by monitoring increases in fluorescence due to oxidation of H2DCF to dichlorofluorescein (DCF). Images were obtained prior to addition of tBH to assess autooxidation and laser-induced changes in fluorescence emission of H2DCF. After a stable baseline was obtained, tBH (0.5 µM) was added following aspiration of the recording chamber solution. Seven additional images were then obtained, 1 min apart. Fluorescence intensity was estimated by delineating the area of the image corresponding to NIH 3T3 cells (as corroborated by simultaneous phase contrast imaging).

Thiobarbituric acid-reactive substances (TBARs) were determined as an independent measurement of lipid peroxidation. Cells were grown to confluence and exposed to tBH (0.5 µM; 4 h). Samples were removed from medium at 20-min intervals and evaluated for malondialdehyde production using a spectrophotometric assay for thiobarbituric acid-reactive substances (23). Cells were trypsinized and protein determined as above.

To determine intracellular antioxidant activity toward phenoxyl radicals, we quantified the ability of cellular homogenates from NIH3T3/MT and TM cells to quench an in vitro generated phenoxyl radical using our recently described method (24). Briefly, oxidative attack on the phenoxyl moiety of etopoide by tyrosinase in air-saturated PBS (pH 7.4) generated phenoxyl radicals that could be measured by laser-induced changes in fluorescence due to oxidation of H2DCF to dichlorofluorescein (DCF). Images were obtained prior to addition of tBH to assess autooxidation and laser-induced changes in fluorescence emission of H2DCF. After a stable baseline was obtained, tBH (0.5 µM) was added following aspiration of the recording chamber solution. Seven additional images were then obtained, 1 min apart. Fluorescence intensity was estimated by delineating the area of the image corresponding to NIH 3T3 cells (as corroborated by simultaneous phase contrast imaging).

TBARs were determined as an independent measurement of lipid peroxidation. Cells were grown to confluence and exposed to tBH (0.5 µM; 4 h). Samples were removed from medium at 20-min intervals and evaluated for malondialdehyde production using a spectrophotometric assay for thiobarbituric acid-reactive substances (23). Cells were trypsinized and protein determined as above.

MT as an Antioxidant in NIH 3T3 Cells

Results

Metallothionein Overexpression and Subcellular Localization—In native NIH 3T3 cells, MT content was approximately 0.1 µg/mg protein, which was similar to that in cells transfected with either pSV2neo alone or pBPVGRPMT (Fig. 1). In contrast, there was a 4-fold increase in metallothionein content after transfection with pBPVGRPMT. Total cellular
and protein thiols were significantly greater in NIH3T3/MT than TM cells, but glutathione levels were similar in either cell (Table I). The increase in MT content was confirmed by a quantitatively greater immunoreactive MT present in NIH3T3/MT (Fig. 2B) than NIH3T3/TM (Fig. 2A) cells. Moreover, the increase in metallothionein appeared to be primarily localized to the cytoplasm as ascertained by confocal microscopy (Fig. 2).

Because of a previous report (26) of MT nuclear translocation in hepatocytes during S phase, we examined the distribution of MT in cells transgressing S phase using BrdUrd labeling and an anti-BrdUrd antibody. No evidence of nuclear MT was seen in cells not treated with BrdUrd (data not shown).

MT in cells transgressing S phase using BrdUrd labeling and an anti-BrdUrd antibody. No evidence of nuclear MT was seen in cells not treated with BrdUrd (data not shown).

**Cellular Sensitivity to tBH—Overexpression of MT produced a phenotype that was resistant to both CdCl₃ (Fig. 3A) and tBH (Fig. 3B). The concentrations of CdCl₃ or tBH required to produce 50% decrease in cell viability in NIH3T3/MT cells was 2-3 fold greater than that needed with NIH3T3/TM cells (p < 0.05). The sensitivity of untransfected NIH 3T3 cells and pSV2neo alone cells to CdCl₃ or tBH was similar to NIH3T3/TM cells (data not shown), indicating that the transfection procedure did not affect cellular sensitivity. While colorometric assays provide a rapid method for determining cellular sensitivity to toxins, they are frequently insensitive. Thus, to quantify more precisely differences in the sensitivity of the transfected cells to oxidants, we next performed clonogenic assays on NIH3T3/TM and MT cells after a brief exposure to tBH. Based on the concentration of tBH required to reduce to 50% survival of NIH3T3/TM (0.03 mM) and NIH3T3/MT (0.18 mM) cells (Fig. 4), overexpression of MT enhanced resistance 6-fold. Untransfected or pSV2neo transfected alone were sensitive to tBH in a manner similar to NIH3T3/TM cells (data not shown).

**DNA Damage—**We performed alkaline elution measurements for DNA integrity 1 h after cellular exposure to a range of tBH concentrations to determine if metallothionein overexpression decreased sensitivity to DNA damage. In Fig. 5, we show that 0.6 mM tBH caused detectable DNA strand scissions within 1 h, but the damage to NIH3T3/MT cells caused by 0.6 mM tBH was no greater than that to NIH3T3/TM cells. Averaging results from three independent experiments, radiation equivalent DNA damage induced by 0.6 mM tBH was 5.09 ± 152 versus 6.19 ± 68 (p = 0.54; not significant) Gy for NIH3T3/TM compared to MT cells, respectively.

**Antioxidant Activity—**Antioxidant activity of cells was assessed by changes in H₂DCF fluorescence, and a typical example is shown in Fig. 6. Prior to tBH, no difference was seen in the DCF signal of NIH3T3/TM (Fig. 6A) or NIH3T3/MT (Fig. 6C). A rapid increase in intracellular oxidant levels was noted in both cell types after 0.5 mM tBH as assessed by an increase in DCF fluorescence, but the oxidant burden was greater in NIH3T3/TM (Fig. 6B) than NIH3T3/MT (Fig. 6D) after tBH exposure. A summary of DCF fluorescence (normalized to pretBH levels) in NIH3T3/TM and TM cells exposed to 0.5 mM tBH is shown in Fig. 7. There was significantly less antioxidant activity in NIH3T3/TM cells than MT cells as assessed by DCF fluorescence.

The antioxidant properties of MT against tBH were further assessed by measuring the emergence of TBAEs. Within 2 h of tBH (0.5 mM) exposure, there were TBAEs present in NIH3T3/TM but not in NIH3T3/MT cells (Fig. 8). The delay in TBAEs formation in NIH3T3/MT cells lasted approximately 1 h. At all times measured, NIH3T3/TM cells consistently had more TBAEs in the medium compared to NIH3T3/MT cells. There was no significant appearance of TBAEs in the medium in the absence of tBH treatment (data not shown).

To probe further the functional antioxidant properties of MT, we examined the ESR spectra of etoposide phenoxyl radical generated by tyrosinase-catalyzed etoposide oxidation in the presence of NIH3T3/TM (Fig. 9A) and NIH3T3/MT (Fig. 9B) cell homogenates. The appearance of the ESR signal with characteristic features of etoposide phenoxyl radical was apparent before addition of cell homogenate, and each cell type immediately quenched the signal. The kinetics of the regeneration of the phenoxyl radical hyperfine ESR signal caused by cellular homogenates provides an estimate of endogenous reductants. That the overexpression of MT produces a functional increase in intracellular antioxidant activity was demonstrated by the differential lag period in ESR signal regeneration (see Fig. 9 and Table I) between NIH3T3/TM cells (14.0 ± 0.5 min) versus NIH3T3/MT cells (22.0 ± 1.0 min; p < 0.05; n = 6).

**DISCUSSION**

Oxidative stress is the result of an imbalance between the production of reactive oxygen species including superoxide anion, hydrogen peroxide and hydroxyl radical, and cellular antioxidant defense mechanisms. Reactive oxygen species are produced during respiration, inflammation, and other cellular metabolic events; in many cases, the metal-catalyzed production of hydroxyl radical is considered critical toward oxidant damage to DNA, proteins, and lipids. Cells have developed elaborate networks to deal with potentially damaging radicals including enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidant systems. In this latter regard, the non-protein thiol, glutathione (GSH), has been studied extensively (27). Much less attention has been directed to protein thiols, such as metallothionein. Metallothionein is particularly interesting because expression of MT is readily inducible (2). Transcriptional activation occurs via heavy metals, as well as cytokines, hormones, and stress; thus, metallothionein levels increase in cells and tissues during conditions known to accompany elevations in reactive oxygen species (4).
Fig. 2. Immunolocalization of metallothionein in NIH3T3/MT (panel A) and NIH3T3/MT (panel B) cells. Transfected NIH 3T3 cells were fixed, permeabilized, and incubated with a rabbit anti-human metallothionein antiserum and then a Cy3 Fluorolink-conjugated, anti-rabbit secondary antibody (see "Experimental Procedures"). Cells were also simultaneously labeled with mouse monoclonal antibody to BrdUrd and BrdUrd co-localized with anti-human metallothionein antibody. Cells were examined with a confocal microscope, and fluorescence intensity was displayed by pseudocolor imaging.

Fig. 3. NIH 3T3 cells that overexpress metallothionein after gene transfer (NIH3T3/MT, open squares) are less sensitive to the acute toxic effects of brief exposures to tBH than control (NIH3T3/M, open diamonds) cells. Cell viability was ascertained by colorimetric assay (see "Experimental Procedures") and normalized to percent of control. In panel A, values are mean ± standard deviation of three separate experiments (or mean of two experiments). In panel B, values are mean ± S.E. of 10 separate experiments (each performed in triplicate). Significant (p < 0.05) differences between NIH3T3/MT and NIH3T3/M cells are indicated by an asterisk.

Most previous studies addressing the antioxidant properties of metallothionein have used inducers such as zinc or other heavy metals, which are promiscuous, affecting a host of genes (28). For example, Chubatsu and Meneghini (8, 12) suggested the protection against H$_2$O$_2$ afforded by cadmium and/or zinc pretreatment is associated with heavy metal effects on other endogenous nonprotein thiol antioxidants. In the current study, we used direct gene transfer in NIH 3T3 cells (Fig. 1) to enhance metallothionein expression and generate a population of cells resistant to the cytotoxicity (Figs. 3B and 4) of the membrane permeant oxidant tert-butyl hydroperoxide. This oxidant is particularly attractive as a model because its cellular actions and fate are well studied (29). tert-Butyl hydroperoxide forms alkoxyl and peroxy radicals through an iron-catalyzed process that cause both cytoplasmic and nuclear damage leading to cell death (14). In hepatocytes, tBH lethality has been linked to lipid peroxidation (14) rather than to DNA single-strand lesions (30). Similarly, we saw no protection against the DNA single-strand breaks with metallothionein overexpression, although cells were clearly protected against the cytotoxic actions of tBH. Thus, metallothionein appears to interfere with the duration of oxidants or the subsequent propagation of free radical reactions rather than the production of primary oxidants or their inactivation.

We used three methods to demonstrate an increase in antioxidant activity in the transfected population: (a) spectrophotometric detection of thioarbituric acid-reactive substances released in the cell culture medium, (b) fluorescence imaging of H$_2$-DCF oxidation in live cells, and (c) cell homogenate quenching of phenoxyl radicals estimated by ESR. The thioarbituric acid assay employed detects free malondialdehyde and is routinely used as an estimate of lipid peroxidation. We noted a clear decrease in appearance of TBARs (Fig. 8) in NIH3T3/MT
versus NIH3T3/MT cells treated with tBH. Despite the obvious differences in TBARs, malondialdehyde can be generated from non-nitro sources and other endogenous aldehydes also can interfere with this assay (34). Moreover, the assay is relatively insensitive and considerable incubation time is required for detectable levels to appear even in cell culture. Therefore, we used an additional highly sensitive technique to detect intracellular oxidants by quantifying the fluorescent changes in DCF after tBH treatment. We noted that tBH caused a prompt and uniform increase in intracellular oxidants in both NIH3T3/MT and NIH3T3/IMT cells (Fig. 6), but the antioxidant activity was greater in NIH3T3/MT cells than NIH3T3/IMT cells since the relative changes in fluorescence were significantly less in the former (Fig. 6). Moreover, the magnitude of the DCF signal displayed little intercellular heterogeneity (Fig. 6). Thus, DCF and TBAR are highly suggestive of an antioxidant role for metallothionein.

Although the mechanism by which metallothionein may act as an antioxidant is unclear, an attractive hypothesis is that metallothionein serves as an expendable target for free radicals (31). Thus, we used a well defined phenoxyl radical generating system (tyrosinase and etoposide) coupled with ESR to evaluate quenching of phenoxyl radicals by homogenates enriched with metallothionein. The lag time before reappearance of the signature ESR signal for phenoxyl radical was previously shown to be independent of the presence of transition metals (24) but was well correlated with total intracellular thiols and poorly correlated to either cell number or total cellular protein (data not shown). In our study homogenates of NIH3T3/IMT cells were significantly more competent than NIH3T3/IMT cells in directly quenching phenoxyl radicals (Fig. 9; Table I). Thus, the collective results of three distinct assays indicate a functional antioxidant role for metallothionein in NIH 3T3 cells.

Recent studies have revealed an antioxidant functionality for metallothionein in lower eukaryotes. S. cerevisiae strains lacking CuZn-superoxide dismutase fail to grow on agar containing the respiratory carbon chain source, lactate, because of their enhanced sensitivity to oxidative injury (13). Expression of either yeast or simian metallothionein after DNA transfer in the presence of copper protects these mutants against oxygen free radical injury (13). Further support for an antioxidant role for copper metallothionein was provided by demonstrating that purified Cu(I)-metallothionein but not other metal species of metallothionein (see below), inhibited the in vitro autoxidation of 6-hydroxydopamine. Interestingly, metallothionein did not substitute for superoxide dismutase in protecting these mutants against either hyperoxia or paraquat (13), so the precise mechanism responsible for protection against the oxidant damage caused by lactate growth has not been established.

Metallothionein may exist in the cytoplasm and/or the nucleus (26), and its subcellular distribution may be critical in determining its antioxidant function. In the current study, we describe a population of cells that have exclusively elevated cytoplasmic expression of metallothionein after gene transfer (Fig. 2A). These cells were resistant to the cytotoxic (Fig. 4) but not the nucleus-damaging (Fig. 5) effects of tBH. The dissociation between cytoplasmic and nuclear effects of tBH have previously been demonstrated (9, 30), and we assume metallothionein must be present within the nucleus to protect against DNA single-strand breaks by tBH. This concept is supported by the recent observation that a clone of V79 cells that had a high nuclear level of metallothionein were resistant to H2O2. In this same study (32), antisense oligonucleotides were used to lower nuclear metallothionein and restore the sensitivity of cells to

Fig. 5. Typical result of DNA scission strand break in cells overexpressing metallothionein (NIH3T3/MT, open circles) or control (NIH3T3/IMT, closed circles) cells after exposure to 0.6 mM tBH. Without tBH added to the medium, there was no detectable DNA scission strand breakage (Control). DNA damage to either cell type was similar after tBH as shown by the comparison of intact [14C]DNA remaining to internal standard (e.g. [3H]) DNA standard after irradiation.

Fig. 6. Effect of tBH on DCF fluorescence in NIH3T3/IMT and NIH3T3/MT cells. Subcultures of NIH3T3/IMT and NIH3T3/MT cells were grown on 31-mm coverslips, loaded with H2-DCF, and exposed to 0.5 mM tBH. Upper panels (A and B) and lower panels (C and D) are from NIH3T3/IMT and NIH3T3/MT cells, respectively. Pseudocolor images of DCF fluorescence are shown before and at 2 min after tBH exposure. Relatively diffuse increases in DCF fluorescence were noted in all cells after tBH, but the intensity was greater in NIH3T3/IMT versus NIH3T3/MT cells.

Fig. 7. Change in DCF fluorescence after tBH in NIH 3T3 cells. DCF fluorescence was quantified from laser imaging at time zero and again 1 min prior to administration of tBH. Images were then obtained every minute for a 7-min interval, and changes in fluorescence were normalized to the initial determination. A total of 8–10 cells/field were analyzed in this fashion, and six fields from six different coverslips were collectively analyzed. Data are mean ± standard error.
creases in nuclear metallothionein expression with zinc enhanced the resistance of this clone to H2O2-mediated nuclear damage. Failure to see protection against H2O2 cytotoxicity in NIH3T3/MT cells after transfer of the human metallothionein-IIa gene might be due to low cytoplasmic localization in these cells (33). We do not understand the factors that direct metallothionein into different subcellular compartments.

As noted above, metal speciation of metallothionein may be important in its potential as an antioxidant. Cu-MT was singularly protective against oxidant stress in mutant yeast lacking CuZn-superoxide dismutase (13). Others have noted that cadmium/zinc metallothionein can actually induce DNA strand breaks in vitro (35). Since we have little information about the metal speciation of metallothionein after gene transfer, this unknown factor could influence the antioxidant function of this protein thiol in our studies.

In conclusion, this report shows an antioxidant role for metallothionein in mammalian cells replete with their normal antioxidant defense mechanisms. Depletion or destruction of superoxide dismutase (13) or GSH levels (36) are not mandatory for physiological amounts of metallothionein to protect cells against free radicals produced by exposure to the membrane permeant TBH. Despite a demonstrable decrease in oxidant burden measured by fluorescence imaging and spectrophotometric detection of thiobarbituric acid-reactive species, the level of single-strand DNA lesions was unaffected and failed to relate to cytotoxicity. Thus, the subcellular location of metallothionein may be important for its antioxidant role.

Acknowledgments—We acknowledge contributions by Dr. Weili Weng and the excellent technical assistance of William Allan and Joseph DeFilippo. We are also grateful to Dr. Kathryn A. Morton (Department of Radiology, University of Utah School of Medicine, Salt Lake City, UT) for the generous gift of pBPVGRPM and pBPVGRPTM.

REFERENCES