Volume 293, number 1,2, 199–203 FEBS 10425 November 1991

Overproduction of human Mn-superoxide dismutase modulates paraquat-mediated toxicity in mammalian cells*

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Received 5 September 1991; revised version received 7 October 1991

Manganese superoxide dismutase (MnSOD) is a nuclear encoded mitochondrial matrix enzyme that functions to scavenge superoxide radicals. The human MnSOD cDNA under the transcriptional control of a human β-actin promoter was introduced into mouse C3H10T$_{\frac{1}{2}}$ cells by cotransfection with a recombinant plasmid containing the Neo^R selectable marker. C3H10T$_{\frac{1}{2}}$ transformants (C3H-SOD) were obtained that expressed high levels of authentic enzymatically active human MnSOD. Overexpression of the MnSOD gene did not affect the protein levels of CuZnSOD, catalase (CAT), or glutathione peroxidase (GPX) in the transformants. Treatment of cells with paraquat was less toxic to the C3H-SOD cells than to the control cells. These results are consistent with the possibility that superoxide radicals are mediators of paraquat cytotoxicity.

Superoxide dismutase; Gene dosage; Mitochondrion; Oxidative stress; Paraquat

1. INTRODUCTION

The herbicide paraquat (methyl viologen; 1,1′dimethyl-4,4′-bipyridinium dichloride) has been responsible for a large number of deaths after accidental or intentional ingestion or injection [1]. The toxic effect of paraquat is believed to be mediated by cytotoxic oxygen free radicals that are produced intracellularly during cyclic oxidation and reduction of paraquat by metabolic processes within the cells [2,3].

It has been shown that treatment of cells or animals with increasing concentrations of paraquat leads to increased cellular content of both Mn- and CuZn-containing superoxide dismutases (MnSOD and CuZnSOD), catalase (CAT), and glutathione peroxidase (GPX) [4,5]. MnSOD and CuZnSOD are protective enzymes responsible for maintaining low levels of superoxide radicals within cells. MnSOD is localized mainly in the mitochondrial matrix [6], whereas CuZnSOD is localized in the cytosol [7]. Dismutation of superoxide radicals yields hydrogen peroxide, which is subsequently detoxified by CAT or GPX.

It is generally thought that the toxicity of superoxide radicals stems from their ability to interact with hydrogen peroxide to generate highly reactive singlet oxygen ($^1O_2$) and/or hydroxyl radicals (OH) [7–10]. Thus, it is possible that the observed increases in antioxidant enzyme systems following exposure to agents that generate superoxide radicals are necessary for the protection of mammalian cells.

To examine the role of MnSOD in the defense against paraquat toxicity, human MnSOD cDNA was introduced into mouse C3H10T$_{\frac{1}{2}}$ cells with a recombinant plasmid containing the Neo^R selectable marker. The selected G418 transformants produced authentic, enzymatically active human MnSOD. The effects of MnSOD overproduction on paraquat-induced cytotoxicity in these cells was then examined.

2. MATERIALS AND METHODS

2.1. *Construction of recombinant plasmid expressing human MnSOD*

To construct the human MnSOD expression vector, the human MnSOD cDNA [11] fragment flanked by the *Eco*RI restriction site was initially blunt-ended with mung bean nuclease, ligated to *Sal*I linkers followed by digestion with *Sal*I, and then inserted into the *Sal*I site of the human β-actin expression vector pHβAPr- [12] (generously provided by Dr. Larry Kedes of the University of Southern California, Los Angeles, CA). All recombinant DNA procedures were performed according to the methods described by Maniatis et al. [13].

2.2. *Transfection and selection of cell lines*

The mouse embryonic fibroblast C3H10T$_{\frac{1}{2}}$ clone 8 was maintained in Eagle's basal medium (BME) supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin. Subconfluent cultures in 100-mm dishes were cotransfected with the MnSOD expression plasmid (10–20 μg) and the pSV2-Neo^R plasmid [14] at a molar ratio of 10:1 by using Lipofectin (BRL). After 48 h, G418 (Gibco) at 400 μg/ml was added to the cells for selection. G418-resistant clones were maintained in complete medium. Control cells were transfected with pSV2-Neo^R

*Preliminary results of this study were presented at the Eighty-second annual meeting of The American Association for Cancer Research, 1991.

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alone and maintained under identical conditions. For detection of MnSOD, cells were grown to confluence in medium without antibiotics.

2.3. *Detection of MnSOD RNA*

Levels of RNA were detected by Northern analysis. Total RNA was isolated by the guanidine isothiocyanate method [15], enriched for poly(A)$^+$RNA [16], size-separated by formaldehyde-agarose (1.1%) gel electrophoresis, and transferred to a nitrocellulose filter. The filter was baked at 80°C and hybridized to a ^{32}P-labeled MnSOD cDNA [17] in 50% formamide at 42°C. The filter was washed twice, for 30 min in 2 × SSC, 0.1% SDS at room temperature, and twice in 0.1 × SSC, 0.1% SDS at 65°C for 1 h.

2.4. *Detection of immunoreactive MnSOD, CAT and GPX protein*

The amount of immunoreactive MnSOD, CAT and GPX protein was measured by a Western blotting procedure as previously described [18]. Briefly, cell homogenates were electrophoresed on a 12.5% polyacrylamide slab gel following pre-treatment with SDS and β-mercaptoethanol at 100°C. Protein from the gel was transferred to a nitrocellulose filter and blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% polysorbate 20 (Tween-20) and 20% FCS. The nitrocellulose filters were then incubated with a 1:5000 dilution of rabbit anti-human MnSOD antibody followed by biotinylated secondary IgG and immunoperoxidase staining.

2.5. *Detection of SOD activity*

The activity of SOD was detected by the nitroblue tetrazolium staining method [19]. Cells were homogenized in 10 mM Tris (pH 7.0)/10 mM dithiothreitol/0.1% Triton X-100 and centrifuged at 10 000 × g for 2 min. Samples were electrophoresed in polyacrylamide gels, consisting of a 5% stacking gel (pH 6.8) and a 10% running gel (pH 8.8).

To visualize SOD activity, gels were first incubated in 2.5 mM nitroblue tetrazolium in H_2O for 15 min followed by 0.028 mM riboflavin/30 mM TEMED/50 mM potassium phosphate (pH 7.8) for 15 min in the dark, washed in deionized water, then illuminated under fluorescent light until clear zones of SOD activity were distinctly evident.

2.6. *Immunochemistry of cultured cells*

Flasks with confluent monolayers were prepared for immunostaining with polyclonal antibodies against MnSOD, CuZnSOD, CAT, and GPX supplied by Dr. Larry W. Oberley as described earlier [20,21]. Staining was performed by the avidin-biotin-peroxidase complex using a universal staining kit (Biogenex Co., Dublin, CA). To preserve the morphology of cells as they were in vitro, cells were not detached from the flasks, but were fixed and stained in situ. The cells were fixed with 10% buffered formalin (3.7% formaldehyde) solution at room temperature for 10 min, rinsed with phosphate-buffered saline (PBS), and postfixed with absolute methanol (−10°C) for 10 min.

Immunostaining was performed following a routine protocol. Cells were rinsed with PBS, and endogenous peroxidase was blocked by incubating for 5 min with a 5% solution of hydrogen peroxide in methanol. The monolayer in each 25-cm^2 flask was divided into 4 areas, 1 for each of the antibodies to antioxidant enzymes and 1 for nonimmune (negative) control serum. Nonspecific antigenic sites were blocked with normal goat serum for 30 min. Excess serum was then removed, and the primary antibodies were incubated overnight at 4°C. The flasks were rinsed and secondary antibody (biotinylated goat anti-rabbit) was applied for 30 min. Label (avidin-peroxidase complex) was added for 30 min. Color was developed by incubating with diaminobenzidine (DAB) (0.25% in PBS), together with a 3% aqueous solution of H_2O_2 for 3–6 min.

2.7. *Assay of paraquat cytotoxicity*

G418-resistant cells and parental cells were grown in BME supplemented with 10% FCS. 24 h before the application of paraquat, cells were seeded in 100-mm dishes (300 cells/dish). Paraquat at various concentrations was applied in quadruplicate, and cells were further incubated. Paraquat was removed after 48 h, cells were rinsed with PBS, and medium without paraquat was added. Cells were allowed to grow for an additional 10–12 days. Cell survival was measured by the ability of C3H10T$_{1/2}$ cells to form colonies in vitro.

3. RESULTS

3.1. *Generation of C3H10T$_{1/2}$ cells that express elevated levels of human MnSOD*

The mouse embryonic fibroblast C3H10T$_{1/2}$ cells were transfected with plasmid pSV-Neo^R, a vector containing a neomycin-resistant gene, or transfected with plasmid pSV-Neo^R plus the human MnSOD expression vector, and then selected for resistance to the antibiotic G418. Five colonies of resistant cells from each transfection were pooled. Integration of the transfected plasmid DNA into cellular DNA was examined by Southern blot analysis [22] (data not shown). RNA blot analysis demonstrated the expression of the human MnSOD sequence in C3H10T$_{1/2}$ cells that were transfected with the human MnSOD vector (C3H-SOD), but not in cells transfected with pSV-Neo^R (C3H-NEO) (Fig. 1).

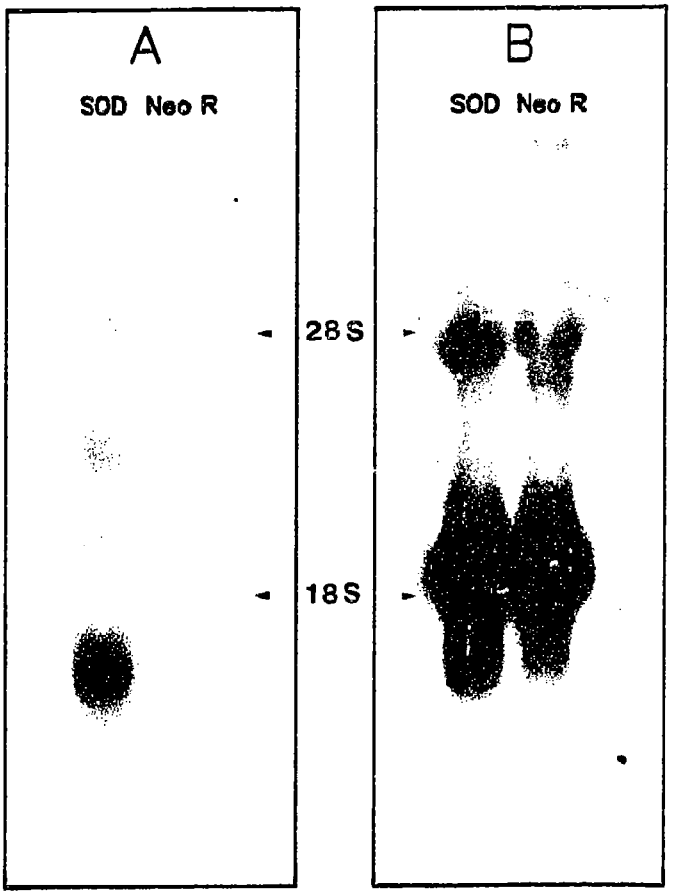

Fig. 1. Northern analysis of cellular RNA. (A) Poly(A$^+$)RNA from C3H-NEO and C3H-SOD cells was separated on a 1.1% formaldehyde agarose gel and, following transfer to nitrocellulose, probed with a ^{32}P-labeled MnSOD cDNA. (B) The same blot was probed with a β-actin.

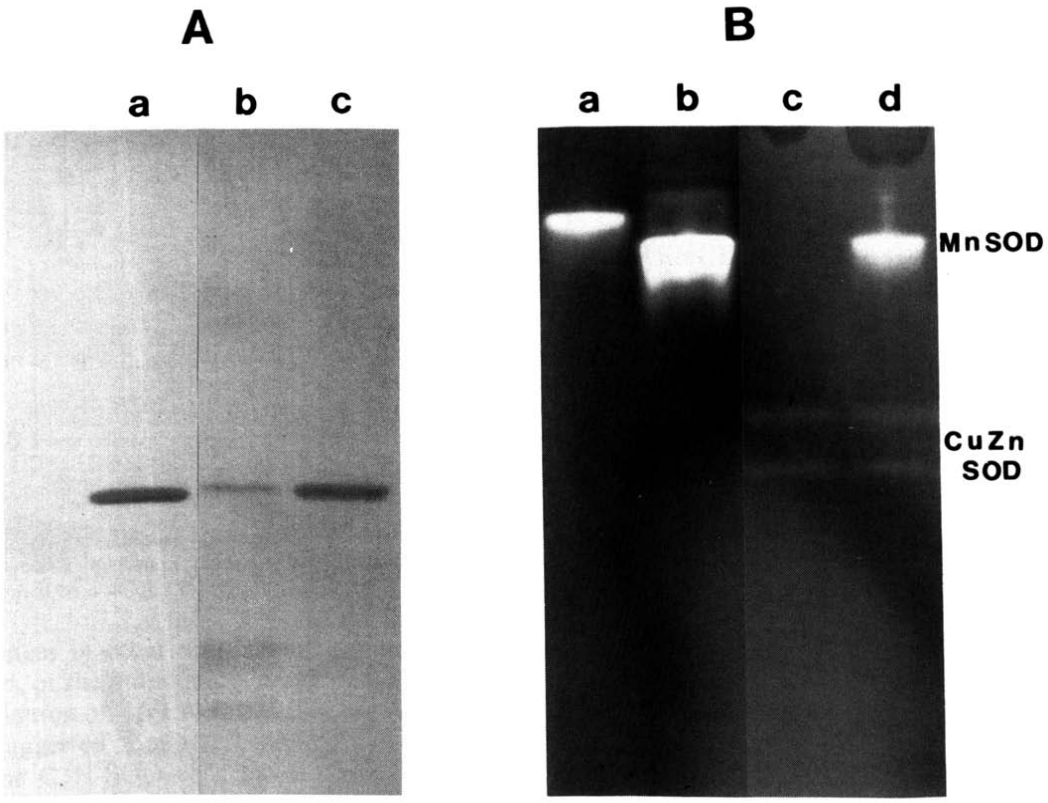

Fig. 2. (A) Western analysis of human MnSOD. Samples were prepared and electrophoresed through polyacrylamide gels, electrotransferred, and then immunologically stained. (Lane a) 1 μg of purified chicken liver MnSOD; (lane b) 250 μg of cell homogenate from C3H-NEO; (lane c) 250 μg of cell homogenate from C3H-SOD cells. (B) Native polyacrylamide gel stained for SOD activity. Samples were electrophoresed through a nondissociating riboflavin gel and stained for SOD activity by the photoinduced NBT reaction. (Lane a) 1 μg purified chicken liver MnSOD; (lane b) 1 μg purified human kidney MnSOD; (lane c) 200 μg of cell homogenate from C3H-NEO cells; (lane d) 200 μg cell homogenate from C3H-SOD cells.

Protein blot analysis using anti-human MnSOD antiserum showed increased MnSOD protein in C3H-SOD cells compared to that in C3H-NEO cells (Fig. 2A). The endogenous levels of CuZnSOD, CAT and GPX were unchanged in C3H-SOD and C3H-NEO cells as examined by Western blot staining (data not shown).

To verify further that the overexpressed MnSOD protein in C3H-SOD cells is active, the SOD activity gel assay was performed. Equal amounts of protein from C3H-NEO cells and C3H-SOD cells were separated on a 10% native polyacrylamide gel, and SOD activity was detected by the nitroblue tetrazolium staining method (Fig. 2B). A species of enzymatically active MnSOD which co-migrated with the purified human MnSOD on the activity gel was found only in C3H-SOD cells.

Immunohistochemical stained cells showed intense granular cytoplasmic staining for MnSOD in C3H-SOD cells, but only slight staining in C3H-NEO and C3H10T$_{\frac{1}{2}}$ cells (Fig. 3).

3.2. *Cellular effects resulting from overproduction of human MnSOD*

If the toxic action of paraquat is mediated through the generation of superoxide radicals [2,3], then the cytotoxic effects of paraquat on C3H-SOD, C3H-NEO, and C3H10T$_{\frac{1}{2}}$ cells should be different. When the cells were treated with paraquat for 48 h and the extent of survival was determined, the C3H-SOD cells were much more resistant to killing by paraquat than either the C3H-NEO or C3H10T$_{\frac{1}{2}}$ cells. The C3H-NEO or C3H10T$_{\frac{1}{2}}$ cells were killed by paraquat to the same extent (Fig. 4).

4. DISCUSSION

The toxic mechanism of paraquat in cells has been linked to the production of excess superoxide radicals [1–4]. If this proposed mechanism for the toxic action of paraquat is valid, then increased intracellular superoxide dismutase should protect cells against paraquat toxicity.

The data presented here demonstrate that an increase in MnSOD activity can protect cells against paraquat toxicity. Thus the role of superoxide radicals in the toxic action of paraquat and a role of MnSOD in the defense against paraquat cytotoxicity are strongly supported by our data. Earlier studies that also support this conclusion include the finding that induction of SOD activity renders bacteria, plants and animals more resistant to paraquat toxicity [4,23,24]. However, in these studies

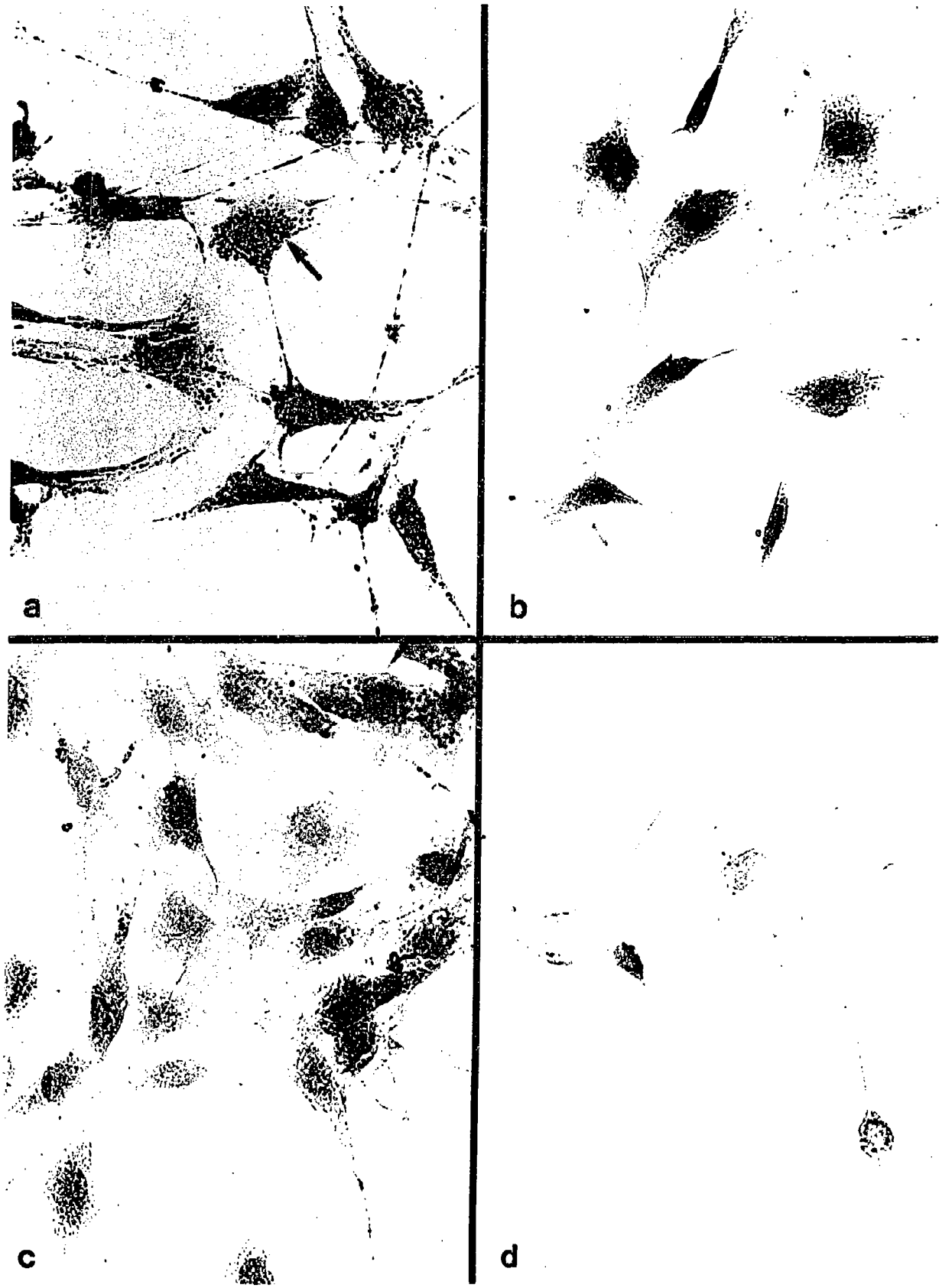

Fig. 3. Immunoperoxidase staining for MnSOD. (a) C3H-SOD with anti-MnSOD antibodies; (b) C3H-NEO with anti-MnSOD antibodies; (c) C3H-10T with anti-MnSOD antibodies, (d) C3H-SOD with nonimmune serum. Magnification ×750. Arrow indicates granular cytoplasmic staining for MnSOD.

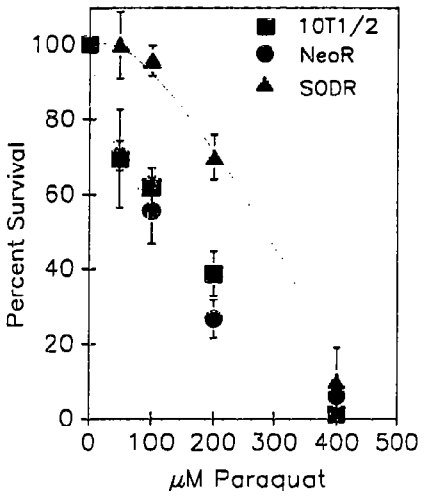

Fig. 4. Dose-response of cell killing by paraquat. Cells were incubated with indicated concentration of paraquat for 48 h. Survival was determined by a colony formation assay.

either the changes in other antioxidant enzymes were not determined, or the induction of SOD was accompanied by an induction of CAT or GPX. Our immunological analysis suggested that overexpression of human MnSOD in the C3H-SOD cells has no effect on the expression of CuZnSOD, CAT, or GPX. In addition, mammalian cells overexpressing human CuZnSOD also show resistance to paraquat [4,24,25]. However, in one study, overexpression of CuZnSOD was accompanied by a decrease in endogenous MnSOD acitivity, and a direct correlation between CuZnSOD activity and resistance to paraquat could not be demonstrated [26]. Thus, our result is the first to demonstrate clearly that MnSOD can be responsible for the protection of mammalian cells from paraquat. Consistent with our results is the study by Wong et al. [27] in which transfection of the MnSOD gene did not cause any change in CuZnSOD, CAT, or GPX levels in the transfected cells and led to cellular resistance to killing by tumor necrosis factor (TNF). Since several lines of evidence implicate oxygen radicals as mediators of TNF-induced cell injury [28–30], the significance of MnSOD in the defense against oxygen radical toxicity in mammalian cells is further confirmed by these latter studies.

The finding that increased MnSOD levels without concurrent increases in CuZnSOD, CAT, or GPX protect cells from the cytotoxicity of paraquat should not be taken as evidence that CAT and GPX are not involved in protecting cells against paraquat toxicity. It has been found that certain clones of cells that exhibit higher CuZnSOD activity are even more sensitive to paraquat [25]. More recent results demonstrate that the levels of endogenous glutathione peroxidase as well as CuZnSOD may also contribute to the tolerance of paraquat by cells [26]. Therefore, the balance of the intracellular redox state may be the key factor in the well-being of cells under oxidative stress.

Acknowledgements: This work was supported by NIH Grants HL-39585 and CA-49797, DOE Grant DE-FG0586ER60464, a grant from the Veterans Administration, and a grant from the Cancer Services, Inc. The authors wish to thank Dr. Larry W. Oberley for the generous supply of all the antibodies used in this study, Dr. Donna Garrison and Nancy Ragland for editing the manuscript, and Darlene Centrall for preparation of this manuscript.

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