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Potential Mechanisms for the Inhibition of Tumor Cell Growth by Manganese Superoxide Dismutase

KWI-HYE KIM, ANA M. RODRIGUEZ, PAULINE M. CARRICO, and J. ANDRES MELENDEZ

ABSTRACT

Studies from many laboratories have shown that overexpression of manganese superoxide dismutase (MnSOD) inhibits the growth of numerous tumor cell types. The inhibition of tumor cell growth can be attributed to the increase in the steady-state levels of H_2O_2 as a result of the increased dismuting activity of MnSOD. Here we demonstrate that overexpression of MnSOD enhances the activity of the superoxide ($O_2^{\cdot -}$)-sensitive enzyme aconitase, decreases the intracellular GSH/GSSG ratio, and dose-dependently inhibits pyruvate carboxylase activity. Thus, alterations in the steady-state concentrations of mitochondrial $O_2^{\cdot -}$ and H_2O_2 as a result of MnSOD overexpression can alter the metabolic capacity of the cell leading to inhibition of cell growth. Furthermore, we propose that MnSOD overexpression can modulate the activity of nitric oxide ($^{\cdot}NO$) by preventing its reaction with $O_2^{\cdot -}$. This hypothesis suggests that the redox environment of the mitochondria can be altered to favor the activity of $^{\cdot}NO$ rather than peroxynitrite ($ONOO^-$) and may explain the enhanced toxicity of $^{\cdot}NO$-generating compounds toward MnSOD-overexpressing cell lines. These findings indicate that therapeutic strategies targeted at overexpressing MnSOD in tumor tissue may be more effective when used in combination with agents that deplete the oxidant-buffering and enhance the $^{\cdot}NO$-generating capacity of the tumor and host, respectively. Antioxid. Redox Signal. 3, 361–373.

INTRODUCTION

Compounds that generate superoxide ($O_2^{\cdot -}$) and other reactive oxygen species have been shown to promote skin tumors in mice, whereas treatment with antioxidants that serve to terminate the chain reactions initiated by reactive oxygen species antagonize this process (58). Manganese superoxide dismutase (MnSOD) levels have also been shown to be abnormally low in malignant tissue as compared with the corresponding normal tissue (17, 46–48, 61). The involvement of MnSOD in tumor suppression may be due to its ability to detoxify $O_2^{\cdot -}$. A number of compelling studies have demonstrated that decreases in MnSOD activity enhance malignant transformation of normal diploid cells, whereas increases in MnSOD levels in transformed cells are associated with a decrease in tumorigenicity of the effected cell. SV40 transformation of human fibroblasts is frequently associated with decreased MnSOD activity. Thorough analysis of SV40 transformed cell lines has shown a strong incidence of transformants containing a deletion of the long arm of chromosome 6 where the MnSOD gene is mapped (5). Thus, it appears that elevated MnSOD is detrimental to the transformed cell. This concept is supported by studies demonstrating that overexpression

Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY 12208.

of MnSOD in numerous transformed cell lines leads to reversion of tumorigenicity *in vivo* (11, 32, 34, 38, 72) or of the malignant phenotype *in vitro* (11, 32–34, 38, 70, 72). St. Clair's group has shown that a number of transformed cell lines display a mutation in the MnSOD promoter that decreases its expression by 50% when compared with the wild-type promoter (69). A recent case study has shown that premenopausal women who are homozygous for a genetic polymorphism in the MnSOD-mitochondrial signal sequence have a fourfold increase in breast cancer risk when compared with those with one or two wild type alleles (2). Thus, a significant and convincing body of evidence suggests that MnSOD may also serve as a tumor suppressor.

The mechanisms underlying the tumor-suppressing ability of MnSOD have not been defined. The currently prevailing theory is that an imbalance in the redox state of the cell leads to an inhibition of cell proliferation. In theory, scavenging of $O_2^{\cdot -}$ by MnSOD leads to an increase in hydrogen peroxide (H_2O_2), and without a concomitant increase in the peroxide-scavenging enzymes, this oxygen metabolite may become toxic. In the mitochondrial microenvironment, excess H_2O_2 would be available to react with Fe^{2+}, contained in multiple Fe-S electron transport enzymes (37), leading either to the production of hydroxyl radical ($HO^{\cdot}$) via the metal-catalyzed Haber–Weiss reaction or of ferryl or perferryl species (23). Li *et al*. have been able to enhance the proliferation of MnSOD-overexpressing MCF-7 cells through the addition of pyruvate (32), which acts as an antioxidant and protects from H_2O_2 toxicity in cultured cells (49). Studies from our laboratory and others have shown that cells can adapt to MnSOD overexpression by compensatory increases in the levels of the H_2O_2-scavenging enzymes, catalase (34, 40) and glutathione peroxidase (33, 38, 40). Several groups have also independently demonstrated that MnSOD overexpression can increase intracellular H_2O_2 levels and that coexpression of either the peroxide-detoxifying enzyme catalase (52) or glutathione peroxidase (36) can reverse this effect. Furthermore, coexpression of either catalase or glutathione peroxidase can reverse the inhibition of cell growth associated with MnSOD overexpression. These findings clearly implicate H_2O_2 as an important mediator of the inhibition of cell growth associated with MnSOD overexpression. Increasing the steady-state concentrations of mitochondrial H_2O_2 without a concomitant increase in the H_2O_2-detoxifying enzymes may lead to the oxidative modification of intracellular proteins and decrease the ability of the cell to respond to oxidizing stress. Studies from this and other laboratories have identified a variety of proteins whose expression or activity is modulated in response to MnSOD overexpression. In addition, the diffusion-limited removal of $O_2^{\cdot -}$ in the mitochondrial microenvironment will alter the activity of other radical species, namely, nitric oxide ($^{\cdot}NO$). This study addresses the consequences of how increased mitochondrial H_2O_2 would lead to the demise of the cell of how altered gene expression in response to MnSOD overexpression would enhance the antitumoral properties of MnSOD, and provides a mechanistic rationale for the increased sensitivity of cells overexpressing MnSOD to $^{\cdot}NO$.

MATERIALS AND METHODS

Reagents

Sodium nitroprusside (SNP) was obtained from Alexis Biochemicals. The Vectastain $Elite_{ABC}$ reagent was obtained from Vector Laboratories (Burlingame, CA, U.S.A.) and the supersignal substrate working solution from Pierce (Rockford, IL, U.S.A.).

Cell culture and transfection

All cell lines were maintained in 25-cm^2 flasks in minimum essential medium containing 10% fetal calf serum, 1,000 U/ml penicillin, 500 μg/ml streptomycin, 1 mg/ml neomycin or 50 μg/ml zeocin in a 37°C humidified incubator containing 5% CO_2. Cell lines used were previously characterized by Melendez and coworkers (40, 52).

In vitro *proliferation assay*

Cells were grown to confluence in 25-cm^2 culture flasks and harvested in 2.0 ml of phos-

phate-buffered saline, pH 7.2, containing 1 m*M* EDTA. Approximately 10,000 cells were seeded onto 96-well culture plates and incubated in a FORMA variable oxygen incubator in an environment of 5% CO_2 in 21% O_2 and treated with the concentrations of SNP indicated. Plates were stained after 24 h using 0.2% crystal violet in 10% ethanol for 10 min, and the excess stain was washed off with water. The stained cells were solubilized with 33% acetic acid, and optical absorbance was measured at 595 nm.

Protein extraction

Cells were grown to confluence in 75-cm^2 culture flasks washed twice and harvested with 2 ml of phosphate-buffered saline, pH 7.2, plus 1 m*M* EDTA. Resuspended cells were centrifuged at 500 rpm for 5 min and washed once. Cell pellets were resuspended in 300 μl of 0.05 *M* potassium phosphate buffer and sonicated for 10 s on ice. The lysate was then centrifuged at 14,000 *g* for 20 min at 4°C and the supernatant collected. The protein concentration of the final supernatant was determined using the bicinchoninic acid protein reagent (Pierce Chemicals Co.).

Mitochondrial fractionation

Approximately 1–5 $\times$ 10^7 cells were harvested as described above, suspended in 10 ml of 1$\times$ isolation medium (230 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES, 1 m*M* EGTA, pH 7.4), and homogenized with 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 163 $\times$ *g* for 80 s. The mitochondria-containing supernatant was layered on 5 ml of 2$\times$ medium (460 m*M* mannitol, 140 m*M* sucrose, 10 m*M* HEPES, pH 7.4) and centrifuged at 1,020 *g* for 10 min. Mitochondria were pelleted from the upper layer of the gradient by centrifugation for 5 min at 4,000 *g*. The pellet was resuspended in 50 m*M* potassium phosphate buffer/0.1 m*M* EDTA, pH 7.8, and protein was extracted as above.

Detection of biotin-containing proteins

Protein extracts were obtained as described above. Twenty micrograms of total protein or 2 μg of mitochondrial protein was loaded to each well of 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and electrophoresed at 100 V for 2 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h and washed briefly with Tris-buffered saline with Tween. The membranes were incubated with Vectastain ABC reagent for 30 min. After washing steps, substrate was added and autoradiographed.

Pyruvate carboxylase assay

Pyruvate carboxylase activity was measured as described by Ballard and Hanson (3). In short, 250 μl of assay reagent (5.0 m*M* $MgCl_2$, 2.5 m*M* ATP, 10 m*M* sodium pyruvate, 10 m*M* sodium [^{14}C] bicarbonate, 0.25 m*M* acetyl-CoA, and 1 U/ml citrate synthase in 100 m*M* Tris-HCl, pH 7.8) was added to 100 μl of mitochondrial fraction in 0.1 *M* Tris-HCl and 0.05% Triton X-100. The mixture was then incubated for 10 min at 37°C. The reaction was terminated by adding 50 μl of 2 *M* HCl. Fifty microliters of reaction mixture was blotted on 2-cm squares of Whatman 3MM filter paper and dried at 90°C for 5 min, and radioactivity was measured in a scintillation counter.

Aconitase assay

Disappearance of *cis*-aconitate at 240 nm was measured with a Spectronic Genesys 5 recording spectrophotometer. Cells were briefly sonicated in 0.15 *M* NaCl buffered with 30 μ*M* triethanolamine-HCl, pH 7.2. Lysates were centrifuged (5,000 *g* for 15 min) and the supernatant immediately assayed for aconitase activity at 25°C in the presence of 0.2% bovine serum albumin. The reaction was started with the addition of 0.2 m*M* *cis*-aconitate, and enzyme activity was determined from the initial reaction rate. An extinction coefficient of 3.41 cm^{-1} mM^{-1} will be used for *cis*-aconitate (26).

Reduced glutathione (GSH) and oxidized glutathione (GSSG) assays

Lysates from sonicated samples were prepared as described above and analyzed for GSH and GSSG by the method of Murphy *et al.* (44). We have developed a modified microplate-based assay of the procedure that al-

lows us to evaluate the GSH/GSSG ratio from a single well of a 24-well plate. In brief, total glutathione (GSH + GSSG) is measured in 95 μl of reaction solution [100 μM NADPH, 5 mM 5,5′-dithiobis(2-nitrobenzoic acid), 1 U of glutathione reductase/ml, 1 mM EDTA, 50 mM K_2HPO_4, pH 7.0] plus 5 μl of sample with appropriate blanks and GSH standards. GSH (without GSSG) was measured in 90 μl of reaction solution (1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 U of glutathione-*S*-transferase/ml, 1 mM EDTA, 50 mM K_2HPO_4, pH 7.0) plus 10 μl of sample, also with blanks and GSH standards.

RESULTS

Effects of MnSOD overexpression on $O_2^{\cdot -}$ and glutathione levels

We have previously reported a decrease in the levels of intracellular $O_2^{\cdot -}$ in MnSOD-overexpressing cells using the $O_2^{\cdot -}$-sensitive fluorophore hydroethidine (40). Redox-sensitive fluorophores are routinely used to evaluate the levels of ROS; however, their specificity has been criticized (53, 54). The inactivation of aconitase has been shown to be an ideal indicator of intracellular $O_2^{\cdot -}$ levels (18, 19). Evaluation of aconitase activity in various cloned cell lines indicates that MnSOD overexpression enhances the activity of aconitase in a dose-dependent fashion, and ·NO donors inhibit this activity (Fig. 1A). These studies further support the idea that MnSOD has dramatic effects on the steady-state levels of $O_2^{\cdot -}$.

The diffusion-limited removal of $O_2^{\cdot -}$ leads to an elevation in intracellular H_2O_2. The H_2O_2 buffering capacity of the cell is largely dependent on the intracellular ratio of GSH/GSSG glutathione. A consequence of the increase in H_2O_2 production in the MnSOD-overexpressing cells may be a decrease in the GSH/GSSG ratio and the cells' redox buffering capacity. Analysis of glutathione indicates that the GSH/GSSG ratio is indeed decreased in MnSOD-overexpressing cells (Fig. 1B).

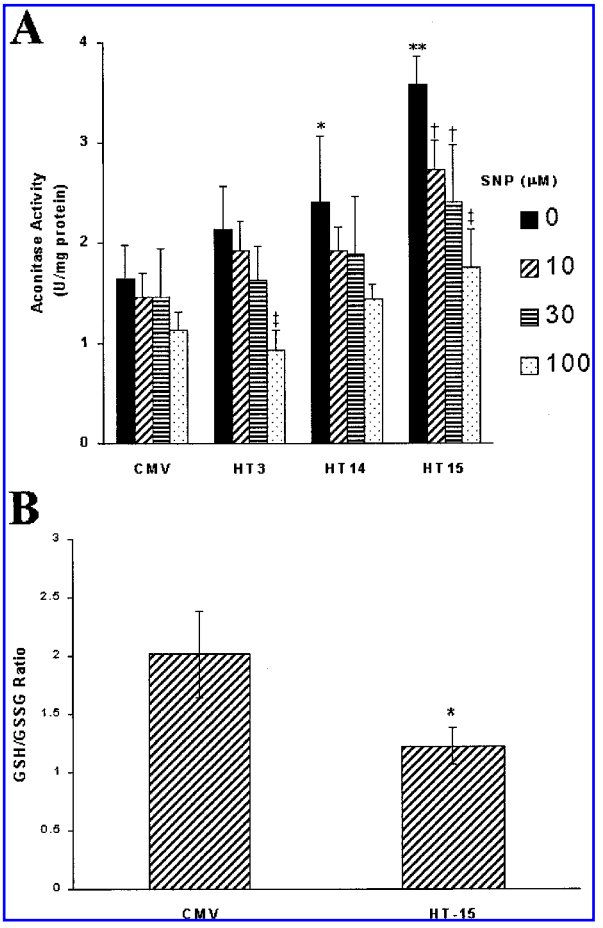

FIG. 1. Analysis of GSH/GSSG ratio and aconitase activities from control and MnSOD-overexpressing fibrosarcoma cells. (**A**) The effect of 12-h treatment in serum-free minimum essential medium with the indicated concentrations of SNP on the aconitase activity of control and MnSOD-overexpressing cell lines. Data are means ± SE from three independent experiments ($n = 3$ per experiment). $^*p < 0.05$, $^{**}p < 0.001$ or $^{\dagger}p < 0.005$, $^{\ddagger}p < 0.001$ when compared with cytomegalovirus (CMV) or untreated control, respectively. (**B**) Total GSH/GSSG ratio from untreated control and MnSOD-overexpressing lines. CMV is the control, and the number following the HT indicates the fold increase in MnSOD activity over control cell lines, *i.e.*, HT15 = 15-fold increase in MnSOD activity. Analysis of both GSH/GSSG ratio and aconitase activity is described in detail in Materials and Methods, and the activities represent the means of three independent experiments where $n = 4$ for each experiment. $^*p < 0.05$. ANOVA with $\alpha = 0.05$ was used for processing the data. Paired t tests were used as posttests.

The effects of MnSOD overexpression on energy production

The overexpression of MnSOD has also been linked to a decrease in intracellular ATP levels (35), and coexpression of catalase can reverse this defect (52). The actual mechanism that leads to the decrease in ATP levels is not clear. We have previously evaluated the contribution of H_2O_2 on mitochondrial function by measuring oxygen uptake in digitonin-permeabilized cell lines (52). MnSOD-overexpressing cells

showed no significant difference in O_2 consumption through complex I, II, or IV when compared with control cells. However, respiration through complex I and II was increased significantly by catalase overexpression. An increase in respiratory chain activity may contribute to the increase in the proliferative capacity of the catalase/MnSOD-overexpressing cell lines by alleviating a metabolic defect associated with MnSOD overexpression that leads to the depletion of ATP in the cell.

The resounding question is what may be causing the decrease in intracellular ATP production in the MnSOD-overexpressing cell lines because no defects in respiratory chain activity were observed in these cells. A clue to how MnSOD may limit ATP production emerged during routine western blotting analysis. During immunoblot analysis of numerous proteins from the MnSOD-overexpressing cell lines, two prominent apparently nonspecific bands were observed at ~70 and 130 kDa. The levels of the 130-kDa protein were decreased in all MnSOD-overexpressing cell lines, whereas the levels of the 70-kDa protein were unchanged (Fig. 2A). Cellular fractionation identified the proteins to be of mitochondrial origin (Fig. 2B). Due to the use of an avidin-conjugated immunodetection system, Vectastain ABC, we predicted that the nonspecific mitochondrial proteins contained biotin. Pyruvate, methyl crotonyl-CoA, and propionyl-CoA carboxylase are the three biotin-containing proteins found in human mitochondria of molecular masses 130, 77, and 72 kDa, respectively. Avidin can interact with naturally occurring biotinylated proteins and produce nonspecific signals (64). The electrophoretic mobilities of the bands were identical to those of the three biotin-containing proteins. The abundant signal of the 60–80-kDa band was actually two independent bands representing the methyl crotonyl-CoA and propionyl-CoA carboxylases (Fig. 2C). The signal intensities of the three nonspecific bands were decreased when blots were blocked with avidin prior to analysis (Fig. 2D). Furthermore, pyruvate carboxylase activity was decreased by 60% in the HT15 cell lines when compared with the cytomegalovirus (CMV) control cells (Fig. 2E). These findings suggest that the signals at 72, 78, and 130 kDa represent biotinylated carboxylases and overexpression of MnSOD down-regulates pyruvate carboxylase, a crucial metabolic enzyme.

·NO, $O_2^{\cdot -}$, and mitochondria

We have previously reported that ·NO donors enhance the MnSOD-dependent suppression of proliferation in fibrosarcoma cells (40). From a therapeutic standpoint, this observation is quite exciting, as ·NO has been shown to be an integral component of the antitumoral immune response. Recent reports from the laboratory of Boveris and co-workers have established that the steady-state concentration of $O_2^{\cdot -}$ is a direct function of the individual mitochondrial concentrations of MnSOD and ·NO (50, 51, 56), and it is these three factors that are most important to the rate of production of peroxynitrite ($ONOO^-$). The investigators have also shown that ubiquinone may act as a scavenger of $ONOO^-$ and limit the degree of nitration of mitochondrial proteins. Thus, it is possible that $ONOO^-$ may be less toxic in the mitochondrial environment than ·NO itself.

We have theoretically estimated what effect MnSOD overexpression may have on $ONOO^-$ production. Under normal steady-state concentrations of $O_2^{\cdot -}$ ($[O_2^{\cdot -}]_{ss}$), the majority of ·NO is converted to $ONOO^-$. MnSOD plays a critical role in the regulation of $[O_2^{\cdot -}]_{ss}$ and subsequently the rate of production of $ONOO^-$. The $[O_2^{\cdot -}]_{ss}$ is determined by the rate of production of $O_2^{\cdot -}$ ($-d[O_2^{\cdot -}]dt$) and by the concentrations of MnSOD and ·NO and their respective rate constants for $O_2^{\cdot -}$ as shown in Eq. 1 (51).

$$[O_2^{\cdot -}]_{ss} = -d[O_2^{\cdot -}]dt/k[\text{MnSOD}] + k\,[\cdot\text{NO}] \quad (1)$$

$$[O_2^{\cdot -}]_{ss} = 1.2 \times 10^{-6}\ M\ s^{-1}/2.3 \times 10^{9}\ M^{-1}\ s^{-1}[0.3 \times 10^{-5}\ M] \times 1.9 \times 10^{10}\ M^{-1}\ s^{-1}\ [5.0 \times 10^{-8}\ M]$$

The above values are based on basal physiological concentration of MnSOD, ·NO, and $O_2^{\cdot -}$ and were obtained from recent reports by Poderoso *et al*. (50, 51, 56). These equations can be used to determine the rate of production of $ONOO^-$ as a function of the steady-state concentration of both $O_2^{\cdot -}$ and ·NO and the rate constant for their diffusion controlled reaction.

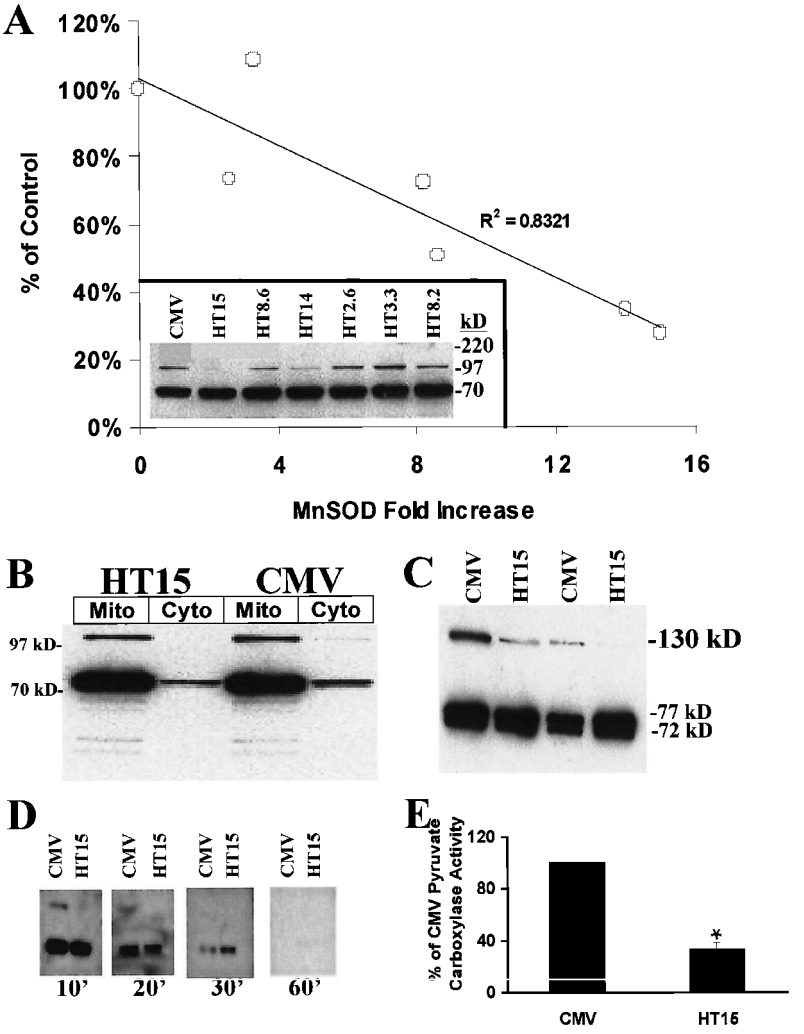

FIG. 2. MnSOD overexpression down-regulates pyruvate carboxylase. (**A**) Protein extracts from control (CMV) and MnSOD-overexpressing cells (HT followed by number indicates fold increase in MnSOD relative to the control cell line) were separated by 10% PAGE and transferred to nitrocellulose membranes. The proteins were detected with Vectastain ABC and the Pierce Supersignal chemiluminescent substrate. Two protein bands were detected at 70 and 130 kDa, and the level at 130 kDa was reduced in MnSOD-overexpressing cells. The protein bands were then quantified using ScionImage data analysis software. The level of the 130-kDa protein was inversely proportional to the level of MnSOD activity when normalized to the level of 70 kDa. (**B**) The 130-kDa protein is exclusively localized in the mitochondrial fraction. Mitochondria were isolated from control (CMV) and MnSOD-overexpressing (HT15) cell lines, and the indicated lysates were analyzed as above. (**C**) The 70-kDa protein represents two bands. Total lysates were analyzed on a 7.5% PAGE, as described above. (**D**) The nonspecific protein signals contain biotin. Proteins were analyzed as described above and blocked with 5% nonfat milk. The membranes were then incubated in avidin and biotin for the indicated time. (**E**) Pyruvate carboxylase activity is decreased in MnSOD-overexpressing cells. Pyruvate carboxylase activity was measured as described in Materials and Methods, normalized to the protein concentration, and expressed as % of control CMV pyruvate carboxylase activity. The data represent four independent experiments with SE $*p < 0.001$. ANOVA with $\alpha = 0.05$ was used for processing the data. Paired Student's t test was used as posttest.

$$+d[\mathrm{ONOO}^-]/dt = k_{\mathrm{ONOO}^-}[\mathrm{O_2}^{\cdot -}]_{ss}\,[^{\cdot}\mathrm{NO}] \quad (2)$$

We have attempted to estimate how the rate of production of $ONOO^-$ is altered as a function of varying both the intracellular concentrations of MnSOD (two- to 32-fold above basal) and $^{\cdot}NO$ in Fig. 3A. It is clear that at low physiological concentrations of $^{\cdot}NO$ ($5 \times 10^{-8}\ M$), the rate of production $ONOO^-$ is directly proportional to the concentration of MnSOD. As the levels of $^{\cdot}NO$ are increased to those observed under pathological conditions ($1–3 \times 10^{-6}\ M$), the dependence of $ONOO^-$ as a function of MnSOD concentrations is diminished. How-

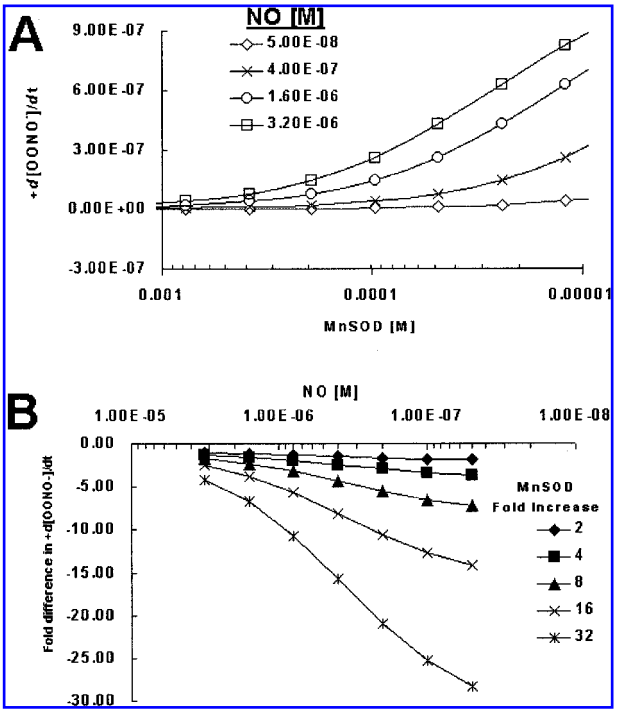

FIG. 3. Theoretical dependence of $ONOO^-$ production on MnSOD and $^{\cdot}NO$ concentrations. (**A**) The rate of production of $ONOO^-$ ($+d[ONOO^-]/dt$) expressed as a function of physiologically relevant MnSOD and $^{\cdot}NO$ concentrations based on Eq. 1 and Eq. 2 and the rate of production of $ONOO^-$. (**B**) The fold decrease in rate of production of $ONOO^-$ expressed as a function of $^{\cdot}NO$ concentrations and the fold increases in MnSOD that are relevant to the present study.

ever, even in the presence of $1–3 \times 10^{-6}\,M$ $^{\cdot}NO$, a 15–30-fold increase in MnSOD levels decreased the rate of production of $ONOO^-$ by three- to fivefold, respectively (Fig. 3b). In support of this hypothesis, studies have demonstrated a decrease in nitrotyrosine levels in transgenic mice overexpressing MnSOD-overexpressing cell lines (30). Thus, the production of $^{\cdot}NO$ from antitumoral effector cells may selectively be toxic to cells with an elevated level of MnSOD.

MnSOD overexpression sensitizes cells to physiologically relevant concentrations of $^{\cdot}NO$ donors. Our calculations would suggest that elevating the levels of MnSOD would enhance the available $^{\cdot}NO$ in the mitochondria leading to the inhibition of cell growth by $^{\cdot}NO$'s ability to inactivate key metabolic enzymes. A potential limitation to the rate of removal of $O_2^{\cdot -}$ by superoxide dismutase (SOD) and the ability of this enzyme to enhance the activity of $^{\cdot}NO$ may be its inhibition by H_2O_2. Hearn et al. (25) have demonstrated that the diffusion-limited removal of $O_2^{\cdot -}$ by MnSOD is inhibited by the H_2O_2. The inhibition of the dismuting function by its product will reduce the catalytic efficiency of overexpressing MnSOD. In support of this hypothesis, cell lines cooverexpressing both MnSOD and catalase in the mitochondria are extremely sensitive to the $^{\cdot}NO$-generating compound SNP (HT15mCAT), whereas cooverexpression of MnSOD and cytosolic catalase (HT15CAT) or mitochondrial catalase (CMVmCAT) alone has no effect on $^{\cdot}NO$ toxicity (Fig. 4). Furthermore, this is a direct cytotoxic effect that occurs in a matter of hours as compared with days in our prior studies. Whether the enhanced cytotoxicity of the coexpression of MnSOD and catalase in the mitochondria is due to the more efficient removal of $O_2^{\cdot -}$ remains to be established.

DISCUSSION

An elevation in the steady-state concentrations of H_2O_2 may have profound effects on the

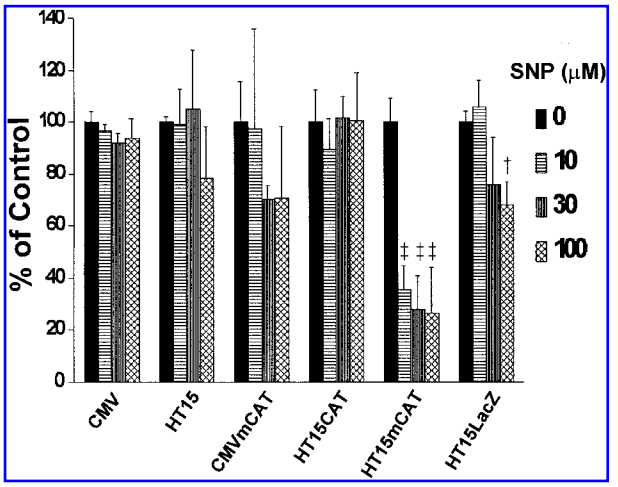

FIG. 4. Coexpression of MnSOD and mitochondrial catalase modulates $^{\cdot}NO$-mediated cell death. Cells were seeded 18 h prior to treatment at a concentration of 10,000 cells/well of a 96-well plate. Cells were then treated overnight with the indicated concentrations of SNP. Cell viability was measured using crystal violet staining. ANOVA with $\alpha = 0.05$ was used for processing the data. Paired *t*-tests were used as posttests. The differences between treatments and their respective controls are indicated: $^{\dagger}p < 0.05$; $^{\ddagger}p < 0.005$.

metabolic capacity of the mitochondria and energy production. An elevation in the steady-state levels of H_2O_2 as a result of MnSOD overexpression must be met with a concomitant decrease in intracellular $O_2^{\cdot -}$ levels. A decrease in $O_2^{\cdot -}$ levels is clearly supported by our finding that the activity of the $O_2^{\cdot -}$-sensitive enzyme, aconitase, is elevated in response to MnSOD overexpression. The ability of MnSOD overexpression to enhance aconitase activity is in agreement with studies on MnSOD knockout mice that show a dramatic decrease in aconitase activity (41, 66). MnSOD overexpression also leads to a dramatic decrease in the GSH/GSSG ratio. This is not unexpected as an elevation in the steady-state levels of H_2O_2 would most likely result in oxidation of GSH. Li *et al.* have also reported a similar effect of MnSOD overexpression on the GSH/GSSG ratio (36). Furthermore, these investigators have proposed that the ability of glutathione peroxidase to detoxify H_2O_2 is strictly dependent on the available GSH, and when conditions stimulate H_2O_2 production and oxidation of GSH, the capacity of the mitochondria to combat this insult would be greatly diminished.

An additional consequence of an elevation in the steady-state concentrations of H_2O_2 is a reduction in the intracellular production of ATP. Numerous studies have reported that the intracellular production of ATP is decreased by treatments with H_2O_2 (1, 13, 27, 68). Furthermore, the ability of chemotherapeutic agents to decrease net ATP production in tumor cell lines is commonly used as an indicator of their efficacy (4, 9, 14, 24, 65). The finding that ATP levels are decreased in MnSOD-overexpressing cells provides a bioenergetic mechanism for the inhibition of cell growth associated with MnSOD overexpression. Mitochondrial enzymes have been shown to be sensitive to inactivation by both reactive nitrogen and oxygen species. MnSOD overexpression may alter the activity of one or many mitochondrial enzymes by a number of different mechanisms, including overproduction of intramitochondrial H_2O_2 that overwhelms the H_2O_2-detoxifying ability of the cell.

The decrease in pyruvate carboxylase levels in response to MnSOD overexpression may have profound effects on the metabolic capacity of the cell. Pyruvate carboxylase catalyzes the first regulated step in the conversion of pyruvate into oxaloacetate, a tricarboxylic acid cycle intermediate that is utilized as the substrate for many biosynthetic processes (28). A decrease in the pyruvate carboxylase activity leads to deficits in gluconeogenesis and tricarboxylic acid cycle activity, leading to an accumulation of alanine, lactate, and pyruvate and subsequent decline in ATP levels. Studies in rat liver mitochondria have shown that the redox-dependent reversible inhibition of pyruvate carboxylase activity by *tert*-butyl hydroperoxide is associated with a rapid increase in GSSG levels and a decrease in the levels of reduced pyridine nucleotides (57). Consequently, intramitochondrial NADH represents the important factor in determining pyruvate carboxylase activity. The overexpression of MnSOD leads to an increase in intracellular H_2O_2 production and may ultimately affect the activity of pyruvate carboxylase by limiting the availability of NADH. In support of this hypothesis, redox-cycling drugs have been shown to enhance the oxidation of NADH and decrease intracellular ATP in PC12 cells. Thus, a decrease in the activity of pyruvate carboxylase may contribute to the metabolic defect associated with MnSOD overexpression.

In addition to perturbing the metabolic capacity of the cell, MnSOD overexpression may also modulate the reactivity of $^{\cdot}NO$. Many of the effects attributed to $^{\cdot}NO$ are actually due to higher-order nitrogen compounds derived from $^{\cdot}NO$. One of the most reactive of these species is $ONOO^-$, which results from the near-diffusion limited reaction of $O_2^{\cdot -}$ with $^{\cdot}NO$ (rate constant $\sim 2 \times 10^{10}\ M^{-1}\ s^{-1}$) (29). The pathophysiological actions of $ONOO^-$ involve its ability to covalently modify macromolecules. The formation of the amino acid derivative 3-nitrotyrosine is generally reflective of nitration reactions catalyzed by $ONOO^-$ and can be detected by specific 3-nitrotyrosine antibodies. *S*-Nitrosylation results from the one-electron reduction of $^{\cdot}NO$ to a nitrosating species, such as NO_x, metal-NO, or $ONOO^-$. The *S*-nitrosothiol that is subsequently formed is predisposed to transfer of the nitrosonium ion (NO^+) to other sulfhydryl centers and may result in enzyme inhibition (59). Nitroxyl anion

(NO^-) has been proposed to be a major contributor to the toxicity associated with $^{\cdot}NO$. NO^- exacerbates H_2O_2-mediated damage and induces single-strand DNA breaks in cultured cell lines (10, 67). Thus, a number of $^{\cdot}NO$-dependent pathways exist that can lead to the ultimate demise of the cell.

A mitochondrial form of $^{\cdot}NO$ synthase has been discovered that positions $^{\cdot}NO$ in close proximity to many known molecular targets (22, 63). Giulivi has demonstrated the inactivation of cytochrome oxidase by nanomolar concentrations of mitochondrial derived $^{\cdot}NO$ (21). It has been demonstrated that atmospheric levels of $^{\cdot}NO$ (120–1,000 ppb) are effective in activating soluble guanylate cyclase only in the presence of SOD (16). It is feasible that levels of $^{\cdot}NO$ that would normally not affect cellular proliferation would become inhibitory in the absence of $O_2^{\cdot -}$. MnSOD may play a critical role in the maintenance of mitochondrial respiration by enhancing the reactivity of $^{\cdot}NO$ toward molecules that would normally be precluded from this chemistry in the presence of $O_2^{\cdot -}$. In addition, hydrophobic interiors of biological membranes have been shown to enhance the biological reactivity of $^{\cdot}NO$ 300-fold relative (39) to aqueous media. The mitochondrion is one such environment where there is a high ratio of surrounding membrane surface to internal aqueous volume. Thus, the mitochondria may be highly susceptible to $^{\cdot}NO$-mediated damage. One adaptation that may benefit tumor-derived or transformed cells lines from this damage is a decrease in MnSOD levels. A decrease in MnSOD levels would decrease the steady-state concentrations of $^{\cdot}NO$ as proposed by our theoretical model.

SOD has also been shown to enhance the toxicity of $^{\cdot}NO$ donors toward ovarian carcinoma (15) and human hepatoma cell lines (20). The increased toxicity is believed to be due to an elevation in H_2O_2 in combination with $^{\cdot}NO$ that subsequently leads to generation of potent oxidants such as the $HO^{\cdot}$ in the presence of trace metals (15). This occurs by a multistep mechanism whereby $^{\cdot}NO$ reduces Fe(III) to Fe(II) followed by the subsequent reduction of H_2O_2 to generate the $HO^{\cdot}$. Studies have also shown that $^{\cdot}NO$ itself may elevate H_2O_2 levels through the inhibition of mitochondrial respiration or via the inhibition of the peroxide-detoxifying enzyme, catalase (7, 15). Both copper/zinc SOD and MnSOD have also been shown to catalyze $ONOO^-$-dependent nitration reactions (55). In the present model system, an elevation in MnSOD levels could possibly lead to an increase in $ONOO^-$-dependent nitration reactions and the covalent modifications of mitochondrial proteins. Murphy and Sies have previously demonstrated that SOD can catalyze the reversible conversion of $^{\cdot}NO$ to NO^- (43). Wink *et al.* have more recently demonstrated that NO^- is orders of magnitude more toxic than $^{\cdot}NO$ (67). NO^--induced toxicity and appearance of site-specific DNA damage are increased in the presence of H_2O_2 by an $HO^{\cdot}$-dependent mechanism (10). MnSOD may potentiate $^{\cdot}NO$ toxicity by any of these proposed pathways.

The GSH/GSSG ratio also plays an important role in protecting against the toxicity of both $ONOO^-$ and $^{\cdot}NO$. Clementi *et al.* have recently demonstrated that $^{\cdot}NO$ can inhibit cellular respiration through complex I by an *S*-nitrosylation reaction (12). The authors demonstrated that the inhibition of complex I was enhanced when glutathione synthesis was blocked and reversed by the addition of GSH and other thiols. The mechanism of this inhibition is reported to be due to the *S*-nitrosylation of critical thiols in the complex I. Glutathione preserves the activity of complex I by competing for $^{\cdot}NO$ and can reverse the inhibitory effect of $^{\cdot}NO$ by transnitrosylation and formation of *S*-nitrosoglutathione (8, 12). MnSOD overexpression has been shown to increase the sensitivity of rat glioma cells to the glutathione synthesis inhibitor, buthionine sulfoximine (71). Thus, the decrease in the GSH/GSSG ratio observed as a result of the increased production of H_2O_2 in the MnSOD-overexpressing cells may contribute in part to the previously reported increased sensitivity of these cells to $^{\cdot}NO$ donors (40).

The ability of activated macrophages to inhibit tumor cell proliferation *in vitro* has long been known to be a consequence of $^{\cdot}NO$ production (45, 60). $^{\cdot}NO$ has been shown to target and inactivate a number of mitochondrial proteins including components of the electron transport system (6, 45, 62) and aconitase (45).

$O_2^{\cdot -}$ generated in the mitochondria may play a role in protecting these sites from reactions with $^{\cdot}NO$. It is unclear how MnSOD enhances the reactivity of $^{\cdot}NO$, but one might speculate that MnSOD may effectively remove $O_2^{\cdot -}$ and decrease the amount of $ONOO^-$ generated, which in the mitochondrial environment may be less toxic to the cell. It is also possible that MnSOD itself may be toxic to the cell in the presence of $^{\cdot}NO$-generating compounds by its ability to catalyze $ONOO^-$-dependent nitration reactions (55). However, we do not believe this to be the case as dihydrorhodamine 123 is also a sensitive indicator of $ONOO^-$ in cells (31, 42) and the levels of its oxidized form are diminished in the MnSOD-overexpressing cells (40).

In summary, increasing evidence indicates that MnSOD plays an important role in regulating tumorigenicity. The exact mechanism for the regulation is unclear. We have previously demonstrated that the effect of MnSOD is clearly oxygen-dependent and that MnSOD enhances the toxicity of the antitumoral immune effector molecule, $^{\cdot}NO$. In this study, we have provided further evidence for the enhanced removal of $O_2^{\cdot -}$ by MnSOD and the increased production of H_2O_2 leading to the decrease in the GSH/GSSG ratio. We have provided a potential mechanism for the metabolic defect that is associated with MnSOD overexpression, and that is loss of pyruvate carboxylase activity. In addition, we have theoretically estimated what effect MnSOD overexpression would have on the biological activity of $^{\cdot}NO$ and the production of $ONOO^-$ and have provided experimental evidence indicating that the toxicity of $^{\cdot}NO$ is greatly enhanced upon the removal of both $O_2^{\cdot -}$ and H_2O_2. The common decrease in MnSOD levels that is observed in numerous tumors and transformed cell lines suggests that $O_2^{\cdot -}$ may provide a selective proliferative advantage to these cells. Our findings indicate that the overexpression of MnSOD may have a multitude of biologic effects that ultimately lead to the demise of the cell

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ABBREVIATIONS

CMV, cytomegalovirus; GSH, reduced glutathione; GSSG, oxidized glutathione; $HO^{\cdot}$, hydroxyl radical; H_2O_2, hydrogen peroxide; MnSOD, manganese superoxide dismutase; $^{\cdot}NO$, nitric oxide; NO^-, nitroxyl anion; $O_2^{\cdot -}$, superoxide; $[O_2^{\cdot -}]_{ss}$, steady-state concentration of $O_2^{\cdot -}$; $ONOO^-$, peroxynitrite; PAGE, polyacrylamide gel electrophoresis; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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Address reprint requests to:
Dr. J. Andres Melendez
Center for Immunology and Microbial Disease
MC151
Albany Medical College
47 New Scotland Avenue
Albany, NY 12208

E-mail: melenda@mail.amc.edu

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