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Enzymatic Activity Is Necessary for the Tumor-Suppressive Effects of MnSOD

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ABSTRACT

The antioxidant protein manganese-containing superoxide dismutase (MnSOD) has been found to be a new type of tumor-suppressor protein. Overexpression of the cDNA for this gene in various types of cancer via plasmid transfection or adenovirus transduction leads to growth suppression both *in vitro* and *in vivo*. The growth-suppressive effect of MnSOD overexpression has been presumed to be due to the enzymatic activity of the MnSOD protein, but could be due to a number of other mechanisms, including a regulatory effect of the RNA or protein produced. To examine this question, we used site-directed mutagenesis to produce a mutant form of human MnSOD that has a leucine at amino acid 26 in the active site rather than the usual histidine. We demonstrate that plasmid transfection or adenoviral transduction of this mutant MnSOD cDNA leads to a large increase in immunoreactive MnSOD protein, but little or no increase in enzymatic activity. In contrast, overexpression of wild-type MnSOD leads to cells with both increased MnSOD protein and activity. Overexpression of wild-type, but not mutant, MnSOD leads to decreased plating efficiency and growth. These results clearly demonstrate that the tumor-suppressive effect of MnSOD protein is largely due to its enzymatic activity. *Antioxid. Redox Signal.* 8: 1283–1293.

INTRODUCTION

AEROBIC CELLS ARE CONSTANTLY BEING EXPOSED to metabolically or environmentally produced reactive oxygen species (ROS). It has been shown that ROS at low concentrations serve physiologically useful functions, whereas at high concentrations, ROS are toxic. One of the more important ROS is superoxide radical anion $O_2^{\bullet -}$. To regulate the superoxide signaling pathways and to protect against the toxicity caused by species derived from superoxide, cells possess a protein designated superoxide dismutase (SOD). This protein is an antioxidant enzyme and is found in several forms in eukaryotic cells. Manganese-containing superoxide dismutase (MnSOD) is found in the mitochondrial matrix, whereas copper- and zinc-containing superoxide dismutase (CuZnSOD) is found in the cytoplasm, nucleus, and lysosomes. SOD catalyzes the reaction:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [1]$$

The hydrogen peroxide produced by SOD has also been shown to be a signaling molecule as well as to have toxic reactivity at high concentrations. A large number of proteins remove peroxides from cells, including the catalases, glutathione peroxidases, peroxiredoxins, and other peroxidases. The balance between peroxide production and peroxide removal is extremely important in governing the physiologic state of the cell.

The SODs are thought to be necessary for life in oxygen-utilizing cells (23). Knocking out the MnSOD gene in mice is lethal, with death occurring days after birth (17, 20). Knocking out the CuZnSOD gene in mice leads to increased susceptibility to oxidative stress (13). Thus, normal cells and organisms need adequate amounts of SOD to protect against the toxicity of species derived from superoxide radical.

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These facts are hard to reconcile with the observations that cancer cells usually have low levels of antioxidant proteins and yet are obviously viable cells that can proliferate at a high rate. It has been found that in general, cancer cells have low levels of MnSOD, CuZnSOD, and catalase activity when compared with an appropriate normal cell control, which is the cell of origin of the particular cancer cell type (26–31). Glutathione peroxidase levels are more variable, and the peroxiredoxins have not been adequately surveyed yet in cancer cells. Even though the levels of the major antioxidant proteins are low in cancer cells, they are not zero, and the concentrations are obviously enough to protect the cancer cell from whatever levels of endogenous ROS are present.

Even though a large body of literature links free radicals and antioxidant enzymes to cancer, most of the evidence is correlative and does not demonstrate a causal relation. Powerful evidence for a causal relation is that in various model systems, ROS cause cancer; moreover, antioxidants in general, and SOD and SOD-mimetics in particular, inhibit malignant transformation (7, 28, 30). Molecular biologic techniques have been also used to demonstrate an important role for SOD in transformation; overexpression of MnSOD by cDNA transfection led to inhibition of radiation-induced transformation in a mouse fibroblast cell line (39). Recently it was shown that a life-long reduction in MnSOD activity (in transgenic heterozygotic mice with a 50% reduction in MnSOD activity) results in a much higher incidence of cancer (41). Moreover, CuZnSOD homozygous knockout mice have a great increase in the incidence of liver cancer (10).

If antioxidant enzymes are important in cancer, then normalization of the levels of these enzymes should result in reversal of at least part of the cancer cell phenotype. This hypothesis was first suggested by Oberley and Buettner in 1979 (27) and has been tested with regards to SOD in three different ways: (a) elevation of SOD by exposure to a superoxide generator and subsequent isolation of resistant cells (11); (b) addition of liposomal CuZnSOD protein (2); and (c) elevation of SOD, particularly MnSOD, by sense cDNA transfection. Each of these techniques has supported the Oberley–Buettner hypothesis. For brevity, only cDNA transfection is discussed.

The first article using cDNA transfection of MnSOD was published in 1993 (8). In collaboration with Drs. Sue Church and James Grant at Washington University, we demonstrated that the transfection of MnSOD cDNA into cultured human melanoma cells resulted in the loss of the malignant phenotype. The malignant phenotype was tested both *in vitro* by assays such as mitotic rate and growth in soft agar and, more important, *in vivo* by growth in nude mice. All of these tests showed a loss of the malignant phenotype in clones that overexpressed MnSOD by at least fivefold. The most important observations were that in the nude mouse assay, 18 of 18 sites injected with the parental melanoma cell line developed tumors, whereas none of 16 sites injected with high-MnSOD–overexpressing cells developed tumors.

We and others have published articles on many other cancer cell types and one virally transformed cell line, showing that overexpression of MnSOD in each of these cell lines led to suppression of cell growth both *in vitro* and *in vivo*. Growth suppression was observed in human breast carcinoma MCF-7 cells (18), virally transformed WI-38 human lung fibroblasts (42), A172R rat glioma (45), U118 human glioma (46), human oral squamous carcinoma SCC-25 (22), mouse (35, 38) and human fibrosarcoma (24), human prostatic carcinoma DU145 (19), and human pancreatic cancer cells (9, 34). Therefore, in all these tumor types, overexpression of MnSOD led to suppression of at least part of the tumor cell phenotype. Thus, the evidence appears substantial that MnSOD elevation by cDNA transfection can suppress the malignant phenotype in a great variety of tumors. On the basis of this work showing growth suppression and the fact that loss of heterozygosity (LOH) for MnSOD has been found in human melanoma (25) and glioma (21), it has been proposed that MnSOD is a new type of tumor-suppressor gene (6).

These cDNA transfection experiments all have the problem that they do not show that active MnSOD protein is necessary for the growth-suppressive activity. In other words, some other property of the transfected DNA could lead to the tumor suppression, and it might have nothing to do with the enzymatic activity of the MnSOD. To examine this question, one must transfect a MnSOD cDNA that produces a protein that has little enzymatic activity. We have attempted to do this in the following manner. It has been reported that the MnSOD protein has two variants at amino acid 58; either isoleucine (Ile) or threonine (Thr) can be at this position in the protein (4). It is still unclear whether this variation is a polymorphism or is a cancer mutation. Isolated Ile58 protein was found to possess twice the enzymatic activity of the Thr58 form and to be more stable against heat (4). Interestingly, amino acid 58 is not in the active site of the MnSOD protein, but in the region between subunits of the tetramer. We sequenced the cDNA we had been transfecting and found that it contained the lesser-activity Thr58 form. We used site-directed mutagenesis to make the Ile58 form. We then transfected both forms into wild-type MCF-7 cells and isolated overexpressing clones (43). Four clones overexpressing Thr58 MnSOD and eight clones overexpressing Ile58 MnSOD were isolated and characterized. The Ile58 clones had three times the MnSOD-specific enzymatic activity of the Thr58 form. Both forms of the MnSOD had tumor-suppressive activity that was in general proportional to the MnSOD activity. The Ile58 clones had a higher tumor-suppressive effect, apparently because they had higher MnSOD activity. These results suggest that the tumor-suppressive effect of MnSOD is largely due to its enzymatic activity.

As mentioned earlier, the Ile58Thr mutation is found in the contact region between subunits of the MnSOD tetramer. We wanted to verify the findings with this mutant and also wanted to examine the effects of an active site mutant. For these reasons, we constructed a new MnSOD mutant that has low MnSOD enzymatic activity because an amino acid in the active site is mutated. We have tested the tumor suppressive ability of this mutant and found that enzymatic activity is necessary for tumor-suppressive activity.

MATERIALS AND METHODS

Reagents

Nitroblue tetrazolium (NBT), xanthine, xanthine oxidase (XO), diethylenetriaminepentacetic acid (DETAPAC), bathocuproine disulfonic acid (BCS), *N,N,N′,N′*-tetramethyl-ethylenediamine (TEMED), bovine serum albumin (BSA), and

actin primary antibody were purchased from Sigma Chemical Co (St. Louis, MO). Riboflavin and the protein assay solution were obtained from BioRad Laboratories (Hercules, CA). Nitrocellulose membranes were purchased from S&S Co (Keene, NH). LipofectAmine, and the transfection medium Opti-MEM were from Gibco BRL Life Technologies, Co (Grand Island, NY). Bacto-Agar was bought from Difco Co (Sparks, MD). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Cell-culture medium and supplementary materials were obtained from the Hybridoma Facility at The University of Iowa. The pcDNA3 plasmid was obtained from Invitrogen Co (Carlsbad, CA). The chemiluminescent ECL kit and Western blotting Rainbow markers were purchased from Amersham Life Science (Buckinghamshire, England). The primary polyclonal antibodies against human MnSOD and CuZnSOD were developed in our laboratory (44). The human catalase primary antibody was obtained from Athens Research & Technology (Athens, GA). Horseradish peroxidase (HRP) conjugated to goat anti-rabbit IgG was purchased from Chemicon (Temecula, CA).

Cell culture

The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. This cell line was purchased from ATCC at passage 33. The cells were cultured in 95% DMEM/F12 with 5% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. Medium was changed every 4 days, and the cells were subcultured with 0.25% trypsin and 1% EDTA whenever the cultures reached confluence. Mycoplasma was tested at 3-month intervals so only mycoplasma-free cells were used.

MIA PaCa-2 is a human pancreatic undifferentiated adenocarcinoma cell line and was obtained from Dr. Joseph Cullen (Department of Surgery, Veterans Affairs Medical Center, Iowa City, IA). The cells were maintained at 37°C in DMEM supplemented with 10% heat-inactivated FBS and 2.5% horse serum and were negative for mycoplasma.

Adenovirus infection

AdEmpty and AdMnSOD were manufactured at Viraquest Inc. (North Liberty, IA). AdMnSOD was originally prepared by John Engelhardt (16) by inserting the MnSOD gene into the E1 region of an Ad5 E1/partial E3 deleted replication-deficient adenoviral vector (15). The cDNA was under the control of a CMV promoter. The MOI for all experiments was calculated from the plaque-forming units (pfu), which was estimated as 1% of the total particles.

MIA PaCa-2 cells were seeded in tissue-culture plates and allowed to attach for 24 h. Cells were then incubated with serum-free media containing adenovirus for 24 h. AdEmpty was used as a vector control. Adenovirus-containing medium was replaced with complete medium for an additional 24 h before cells were harvested.

Site-directed mutagenesis

MnSOD cDNA mutants were made by the QIAGEN (Valencia, CA) Quikchange Site-Directed Mutagenesis kit. The basic procedure uses a double-stranded DNA vector, which is annealed to two synthetic sequences containing the desired mutation. The two mutagenic primers (sense and antisense) should be between 25 and 45 bases in length; the desired mutation should be in the middle of the primers; the primers should have a minimum GC content of 40%, and terminate in one or more C or G bases; and the two primers should exactly match each other. Then PCR is used to amplify by using pfu DNA polymerase. We prepared the following sample reactions; each sample contained 5 µl 10× reaction buffer, × µl ds DNA template (ranging from 5 to 50 µg), 125 ng each primer, 1 µl dNTP mix, and then distilled water was added to a final volume of 50 µl, and finally 1 µl pfu DNA polymerase was added. Then the following PCR reactions were performed. Cycle 1, temperature 95°C, 30 sec, once; cycle 2, 95°C, 30 sec; 55°C, 1 min; 68°C, 2 min/kb of plasmid length, total 12 cycles for point mutations. In this way a mutated plasmid was generated. The product was treated with *Dpn1* endonuclease, which is specific for methylated DNA. *Dpn1* deletes the original DNA. After temperature cycling, the samples were placed on ice for 2 min to cool the reaction to ≤37°C, and then 1 µl of the *Dpn1* restriction enzyme was added, gently mixed, spun, and each reaction incubated at 37°C for 1 h to digest the parental supercoiled ds DNA (*i.e.*, the nonmutated DNA). Finally, the mutated plasmid was transformed into *Escherichia coli*, and clone selected. 1 µl of the *Dpn1*-treated DNA from each sample reaction was transformed to separate aliquots of the supercompetent cells, the reactions swirled and incubated on ice for 30 min, and then heated for 45 sec at 42°C and placed on ice for 2 min. Finally, 0.5 ml of NZY + broth (10 g NZ amine, 5 g yeast extract, and 5 g NaCl) preheated to 42°C was added, and the transformation reactions incubated at 37°C for 1 h with shaking at 225–250 rpm; then immediately the entire volume of the transformation reaction was plated on agar plates, and the plates incubated at 37°C for >16 h.

Plasmid DNA preparation

MnSOD cDNA was mixed with *E. coli* competent cells and then plated onto LB agar plates (10 g NaCl, 5 g yeast extract, 10 g tryptone, 20 g agar, and 50 mg ampicillin) at 37ºC overnight. On the following day, a single colony was picked into LB medium, which was shaken at 200 rpm at 37ºC overnight until the medium became saturated. This usually took 12–16 h. Plasmid DNA was isolated by using a QIAGEN Plasmid Midi Kit. The bacterial pellet was resuspended in RNase buffer, and then lysed with 0.2 *M* NaOH and 1% SDS, neutralized with 3 *M* potassium acetate and equilibrated with 0.75 *M* NaCl. After separating by gravity flow on a column and centrifugation, the DNA was washed with 1 *M* NaCl and 15% isopropanol, eluted with 1.25 *M* NaCl, precipitated with 0.7 volumes of isopropanol, washed with 70% ethanol, the DNA was again pelleted by centrifugation at 15,000 *g* for 10 min, air dried, and finally redissolved in TE buffer (pH 8). After purification, the DNA concentration was quantitated with a spectrophotometer by measuring the absorbance at 260 nm (OD_{260}). The protein concentration was determined by measuring the absorbance at 280 nm (OD_{280}). DNA with an OD_{260}/OD_{280} ratio >1.5 was accepted, and the concentration of DNA was calculated by the following formula:

$$\text{DNA } (\mu g/\mu l) = \text{Dilution factor} \times 50 \times OD_{260} \qquad [2]$$

The plasmid DNA was checked by restriction enzyme analysis: 1 µl DNA was mixed with 5 U enzymes (*Eco*R1, *Kpn*1). The mixture was incubated at 37ºC for 1 h and electrophoresed in 0.8% agarose at 100 V for 30 min in TBE solution. Under UV light, the size of DNA, which corresponded to inserted MnSOD cDNA, was confirmed. The purified plasmid DNA was stored at –20°C and used for transient and stable transfection.

Transient transfection of MnSOD cDNA into 293 cells by using LipofectAmine

Before transient transfection, the 293 cells were sent to the Hybridoma Facility at The University of Iowa for testing of Mycoplasma contamination. If the testing result was negative, then the transient transfection was carried out. The vector that carried sense MnSOD cDNA used in this experiment was pcDNA3. The pcDNA3 plasmid is a eukaryotic expression vector containing a CMV promoter and a neomycin-resistance gene. The plasmid containing MnSOD cDNA was constructed in our laboratory (18). The cDNA was inserted into the pcDNA3 plasmid between the restriction enzyme *Kpn*1 and *Eco*R1 sites.

A new transfection reagent, LipofectAmine, introduced by Life Technologies Inc., has been shown to be simple, highly reproducible, and efficient compared with some other commonly used procedures such as electroporation, polycations, calcium phosphate, liposome fusion, retroviruses, microinjection, and protoplast fusion. Before transient transfection, the required plasmid and LipofectAmine concentration was determined, and then transient transfection was carried out. The 293 cells were seeded at 2×10^6 cells in 100-mm dishes (Corning, Corning, New York) with the complete growth medium, and incubated at 37°C in a CO_2 incubator for 24 h to obtain 50–80% confluence before transient transfection. The transfection was performed with LipofectAmine according to the manufacturer's recommendations. The transfection medium contained 45 µl of LipofectAmine and 10 µg of plasmid DNA, which were separately diluted in 800 µl of OPTI-MEM I reduced serum medium and then mixed together, incubated at room temperature for 45 min to allow the formation of DNA–liposome complexes, and then the combination diluted into 6.4 ml serum-free medium and overlaid onto the cells. After 5 h at 37°C in a CO_2 incubator, 1 ml of growth medium containing twice the normal concentration of serum was added without removing the transfection mixture. The next day the medium was replaced with fresh, complete medium, and after another 20 h, the cells were harvested with rinsing in phosphate-buffered saline (PBS, pH 7.0) twice, scraping with a rubber policeman into PBS, and then centrifuged at 500 *g* for 3 min. After removing the PBS, the cell pellets were stored at −20°C until use.

Stable transfection of MnSOD cDNA into MIA PaCa-2 cells

Cells (1×10^5) were seeded into six-well plates 24 h before transfection. The expression vector pcDNA3 only and the plasmid-containing human MnSOD cDNA were transfected into MIA PaCa-2 cells by using the LipofectAmine reagent according to the instructions provided by the manufacturer. The MnSOD-containing plasmid (5 µg) and LipofectAmine reagent (10 µl) were mixed and incubated at room temperature for 45 min to allow the formation of a DNA–lipid complex. The same preparation was used for the vector-only control solution. Cells were incubated with these complexes in serum-free medium for 5 h at 37°C and 5% CO_2. After this incubation, 1 ml of growth medium containing twice the normal concentration of serum was added to the cells without removing the transfection mixture. Cells were provided with fresh, complete growth medium 24 h after transfection. At 72 h after transfection, cells were subcultured into G418 (1 mg/ml) selection medium with 1:10, 1:100, and 1:1,000 dilutions, and incubated for 14 days. Resistant colonies were selected and grown in DMEM containing 10% heat-inactivated FBS, 2.5% horse serum, and G418 (400 µg/ml).

Sample homogenization and protein quantitation

Cells used for Western blot or activity analysis were first washed 3 times with PBS, pH 7.0, and then scraped with a rubber policeman into a microcentrifuge tube. The harvested cells were centrifuged at 500 *g* for 3 min. The cell pellets were stored at −20°C until analysis. On the day of analysis, the cell pellets were resuspended in 50 m*M* potassium phosphate buffer (PB), pH 7.8, and sonicated on ice with three bursts of 20 sec each by using a Vibra cell sonicator with a cup horn (Sonics and Materials Inc.) at 30% power. Protein concentration was quantitated by Bradford's method (5) using a Bio-Rad Protein Assay according to manufacturer's instructions.

Sequencing of human MnSOD cDNA by the fluorescent method

Automated DNA sequence analysis was performed in the DNA Core Facility at University of Iowa. In a 0.5-ml Eppendorf tube was placed: 0.5 µg plasmid with human MnSOD cDNA and 4 µl of 0.8 pmol/µl sense or antisense primer (SP6 and T7) in a total volume of 11 µl of distilled water. The sequencing results were analyzed by GCG (Genetics Computer Group) Sequence Analysis Software Package.

MnSOD immunoprotein analysis by Western blotting

The amount of immunoreactive MnSOD protein was measured by the technique of Laemmili (14). In brief, 10 µg of total proteins was denatured with SDS loading buffer at 94°C for 4 min and then separated by electrophoresis on a 12.5% SDS-polyacrylamide running gel with a 4.5% stacking gel in SDS-Tris-glycine running buffer pH 8.8 at 100 V. Proteins were electrotransferred to a nitrocellulose membrane at 100 V for 1 h at 4°C. After blocking with 5% dry milk at room temperature for 1 h, the blot was incubated with a polyclonal anti-rabbit MnSOD (1:1,000), CuZnSOD (1:2,000), or CAT (1:1,000) antibody in 5% milk at room temperature for 1 h. After washing 3 times with TBST (0.02 M Tris buffer pH 7.0 and 0.05% Tween 20), 5 min each, the blots were incubated with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase conjugate at 1:10,000 dilution in TBST for 1 h at

room temperature. The washed blot was then treated with ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, England) and exposed to x-ray film (Eastman Kodak, Rochester, NY).

MnSOD and catalase activity gels

The SOD activity gel assay is based on the inhibition of the reduction of nitroblue tetrazolium (NBT) by SOD (1). The CAT activity gel assay was carried out according to the methods described by Sun *et al*. (40).

MnSOD spectrophotometric activity assay

The standard SOD activity assay using the modified NBT reduction method was described by Oberley and Spitz (32, 33, 37). In this indirect method, xanthine–xanthine oxidase is used to generate $O_2^{\bullet -}$, and NBT reduction is used as an indicator of $O_2^{\bullet -}$ production. SOD will then compete with NBT for $O_2^{\bullet -}$. MnSOD activity was measured in the presence of 5 m*M* NaCN in the mixture to inhibit CuZnSOD activity. One unit of SOD activity was defined as amount of protein required to give half-maximal inhibition of NBT reduction. The activity values were expressed in units per milligram protein (U/mg protein).

Plating efficiency

Plating efficiency was measured as previously described (36). Cells (500–2,000) were seeded in 60-mm dishes, incubated for 14 days to allow colony formation, and then fixed and stained with 0.1% crystal violet. The colonies containing ≥50 cells were scored. The plating efficiency (PE) was calculated as follows: PE = (colonies formed/number of cells seeded) × 100%.

Growth curve

Cells (5,000) of different MOI treatment were plated in triplicate in six-well plates. The growth rate of cells was determined by counting the number of cells with a hemocytometer as a function of time. Cell population doubling time (*Td*) was calculated from the growth rate during exponential growth by the following formula:

$$Td = 0.693(t - t_0)/ln(N_t/N_0) \quad [3]$$

where t_0 is the time at which exponential growth began, t is time in days, N_t is the cell number at time t, and N_0 is the cell number at the initial time.

Statistical analysis

The significance of group differences in MnSOD protein and activity levels was assessed with ANOVA. Comparisons of plating efficiency between groups were performed with Poisson regression. Multivariate ANOVA was used to compare cell growth in the different MOI treatment groups, and cell-doubling times were analyzed with one-way ANOVA. Statistical tests were carried out at the 5% level of significance with the SAS statistical software (Cary, NC). Pairwise group comparisons were performed with the Tukey's HSD procedure to control the overall significance level of each experiment. Tests for mean differences in these experimental replicates were nonsignificant. All Western blots and activity gels were run at least twice to show reproducibility.

RESULTS

The active site of human MnSOD contains His-26 and His-74 from the N-terminal domain of the protein and Asp-159 and His-163 from the C-terminal domain. The fifth coordination site of the manganese in the active site is thought to be occupied by a water molecule (3). We used site-directed mutagenesis to change the histidine (CAC) at amino acid 26 to a leucine (CTC). The change was confirmed with DNA sequencing (data not shown). Wild-type or mutant MnSOD cDNA was inserted into the pcDNA3 plasmid between the restriction enzyme *Kpn*1 and *Eco*R1 sites. The pcDNA3 plasmid is a eukaryotic expression vector containing a CMV promoter and a neomycin-resistance gene. Plasmid with no added DNA was designated Neo and was used as a transfection control.

We used transient transfection to introduce the pcDNA3 plasmid into 293 cells. The 293 cells are primary human embryonal kidney cells transformed by sheared human adenovirus type 5 DNA. Transient transfection was accomplished by using the LipofectAmine reagent according to the manufacturer's recommendations. After transfection, samples were analyzed by Western blotting and activity gel, as shown in Fig. 1. Control untransfected 293 cells (lane 1) or Neo transfected cells (lane 2) showed little MnSOD protein or enzymatic activity. Cells transfected with wild-type (WT) MnSOD showed a large increase in both MnSOD protein and activity (Fig. 1a and b, lane 3). In contrast, cells transfected with the mutant MnSOD H26L demonstrated a large increase in immunoreactive protein, but no increase in activity (Fig. 1a and b, lane 4). This was confirmed by the densitometry of the Western blot and activity gel, as shown in Fig. 2.

To examine these results with another technique, we also performed stable transfection experiments. The 293 cells were again transfected with the pcDNA3 plasmid and then clones isolated that overexpressed either WT or mutant MnSOD. Two WT cell lines (WT5, WT6) and two mutant cell lines (H26L4, H26L8) were studied. Western blotting showed an increase in MnSOD, but not in CuZnSOD or catalase, immunoreactive protein in all clones, whether expressing mutant or wild-type MnSOD (Fig. 3). Conversely, the two wild-type clones showed a large increase in MnSOD enzymatic activity (Fig. 4, lanes 2 and 3 compared with control, lane 1), whereas the mutant clones showed no increase in MnSOD activity (Fig. 4, lanes 4 and 5). All samples had similar CuZnSOD and catalase enzymatic activities (Fig. 4). These results on MnSOD activity with a semiquantitative activity gel were confirmed with the quantitative spectrophotometric activity assay, as shown in Fig. 5a. The 293 cells stably overexpressing wild-type MnSOD showed about a 10-fold increase in MnSOD activity compared with the untransfected control cells. Cells overexpressing the mutant MnSOD demonstrated no changes in MnSOD enzymatic activity. Thus, stably transfected clones overexpressing mutant MnSOD demonstrated a

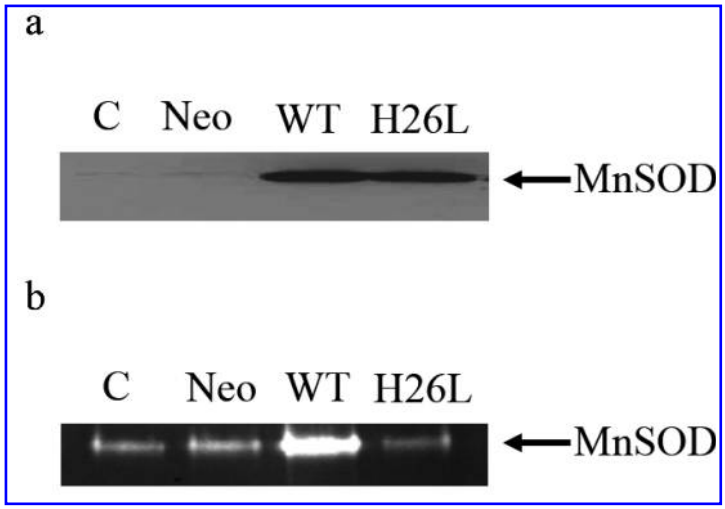

FIG. 1. Mutant MnSODH26L transiently transfected into 293 cells increased MnSOD protein levels and decreased MnSOD activity. (**a**) Cellular protein (10 μg) was separated on a 12.5% SDS polyacrylamide gel, and Western blotting was performed. The expression of MnSOD protein was observed in the ~22-kDa band. Mutant H26L had the same high protein level as did the wild-type transfectant. (**b**) Cellular protein (80 μg) was separated in a 12% native polyacrylamide gel and stained with a solution containing 0.75 mM sodium cyanide to visualize MnSOD activity only. Electrophoresis was performed at 4°C for 3–4 h. Compared with the band of control (no treatment) and vector control (Neo), the activity gel showed a greatly increased intensity of the activity band in the wild-type transfectant and decreased intensity of the activity band in the H26L transfectant.

large increase in MnSOD immunoreactive protein, but no increase in MnSOD enzymatic activity.

To examine the effect of MnSOD overexpression on cell phenotype, we measured the effect on plating efficiency (PE). Plating efficiency is usually higher in cancer cells compared with normal cells, and MnSOD elevation has usually been shown to decrease plating efficiency. In the present study, clones overexpressing WT MnSOD had lower PE compared with control cells (Fig. 5b). In contrast, cells overexpressing H26L actually had higher PE than control cells. Thus, overexpression of the different forms of MnSOD had a substantial effect on the cellular phenotype, and mutant MnSOD did not lead to the decreased PE usually seen with wild-type MnSOD overexpression.

To verify these results, MnSOD was overexpressed by another technique: adenovirus transduction. In addition, we switched to a more malignant cell line, human pancreatic cancer MIA PaCa-2 cells. Both WT and mutant MnSOD adenoviral constructs were made by Viraquest, Inc. The adenovirus expressing wild-type MnSOD has been extensively tested by our laboratory and been shown to express active MnSOD protein. As shown in Fig. 6, increasing the MOI of the adenovirus expressing mutant MnSOD led to increasing

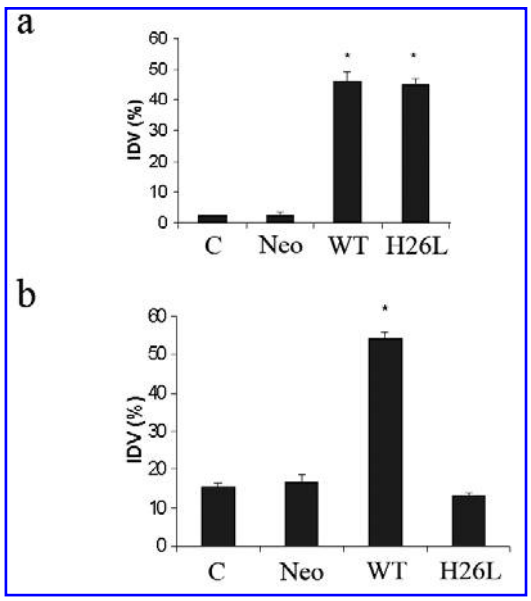

FIG. 2. Densitometric analysis of Western blot and activity gel of MnSOD. (**a**) Densitometric analysis of Western blotting bands showed increases in MnSOD protein levels from wild-type and H26L transfectants. The mean IDV and standard deviations for three separate experiments are shown. *$p < 0.05$ compared with the parental control cell line. (**b**) Densitometric analysis of activity gel bands showed increases in MnSOD activity from wild-type transfectant, but decreases in the H26L transfectant. The mean IDV and standard deviations for three separate experiments are shown. *$p < 0.05$ compared with the parental control cell line.

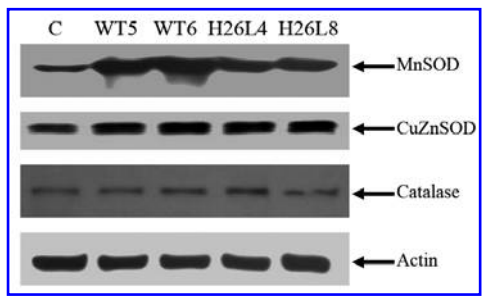

FIG. 3. Stable transfection of human MnSOD cDNA and mutant MnSOD H26L increased protein levels of MnSOD in 293 cells. Cellular protein (10 μg) from each cell line was separated on a 12.5% SDS polyacrylamide gel for Western blotting for MnSOD, CuZnSOD, and catalase. The expression of MnSOD, CuZnSOD, and catalase protein was observed in the ~22-, 16-, and 60-kDa bands, respectively. An increased expression of MnSOD was observed in the ~22-kDa band in the two wild-type transfected cell lines (WT5, WT6), and two mutant H26L-transfected cell lines (H26L4, H26L8), but the CuZnSOD and catalase protein did not change. Actin was used as loading control.

amounts of MnSOD immunoreactive protein. Similar levels of MnSOD immunoreactive protein were found in cells transduced with adenovirus expressing 200 MOI of wild type MnSOD and 25 MOI of H26L (Fig. 7). Thus, the adenovirus expressing mutant MnSOD was much more effective at expressing the protein than the adenovirus expressing wild-type MnSOD. Therefore, to compare the enzymatic activity of cells with equivalent amounts of MnSOD immunoreactive protein, in Fig. 8 again, 200 MOI of the adenovirus expressing wild-type MnSOD was compared with only 25 MOI of the adenovirus expressing the mutant MnSOD. Transduction with adenovirus expressing WTMnSOD, but not mutant MnSOD, led to increases in MnSOD enzymatic activity (Fig. 8). No changes were found in the levels of CuZnSOD or catalase protein (Fig. 7) or activity (Fig. 8) after transduction of adenovirus expressing WT or mutant MnSOD. Adenoviral transduction led to increases in both wild-type and mutant MnSOD protein for as long as 7 days after transduction (data not shown).

We next examined the effect of adenoviral infection on cell phenotype. Adenoviral WTMnSOD inhibited growth, whereas adenovirus expressing mutant MnSOD had much less growth-suppressive effect. At 200 MOI, some effect was seen with empty virus, but 200 MOI of adenovirus expressing wild-type MnSOD suppressed growth more, and adenovirus expressing mutant MnSOD (25 MOI) suppressed growth less than 200 MOI of empty adenovirus (Fig. 9a and b). Similarly, adenoviral transduction of wild-type MnSOD led to a statistically significant reduction in plating efficiency (Fig. 9c), whereas adenoviral transduction of the mutant MnSOD led to no change in plating efficiency. Thus, at similar levels of protein expression, wild-type MnSOD had a growth-suppressive effect, but mutant MnSOD did not. This strongly suggests that the growth-suppressive effect of MnSOD overexpression is due to the enzymatic activity of the MnSOD protein.

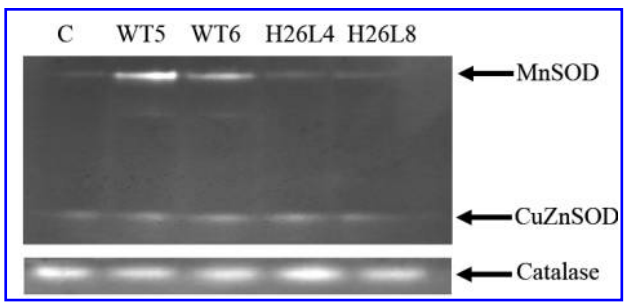

FIG. 4. Stable transfection of human mutant MnSOD H26L decreased activity level of MnSOD in 293 cells. Cellular protein (100 μg) was separated in a 12% native polyacrylamide gel for SOD activity, and 30 μg of cellular protein was separated in an 8% native polyacrylamide gel for catalase activity. Compared with the band of control (no treatment), the activity gel showed a greatly increased intensity of the activity band in the wild-type transfectants (WT5, WT6), and decreased intensity of the activity band in the H26L transfectants (H26L4, H26L8), but the CuZnSOD and catalase activity did not change.

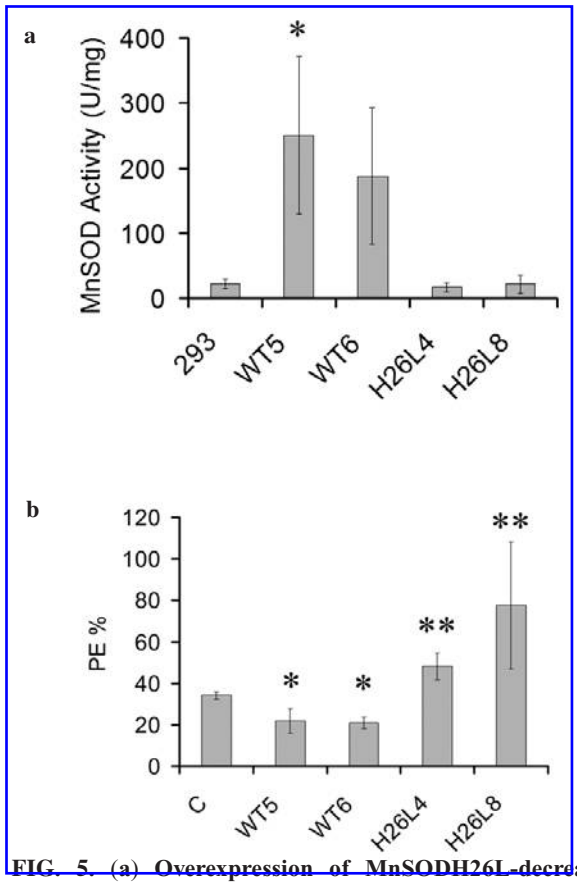

FIG. 5. (a) Overexpression of MnSODH26L-decreased MnSOD activity. The total cellular protein was assayed for MnSOD activity. One unit of MnSOD activity is defined as amount of protein required to give half-maximal inhibition of NBT reduction. Values are expressed as mean ± SEM of three independent cultures. The MnSOD activity was increased by 10- to 12-fold in the MnSOD-overexpressing cell lines compared with the parental cell line (*$p < 0.05$ in line WT5, but not significant in line WT6), but not significantly changed in the mutant MnSOD-overexpressing cell lines (H26L4 or H26L8) compared with the 293 parental cell line ($p > 0.05$). (**b**) Overexpression of MnSODH26L-increased plating efficiency. Cells were seeded into 60-mm dishes at a certain cell number. After 2 weeks' incubation at 37°C, cells were fixed and stained. Colonies containing >50 cells were counted. The plating efficiency (PE) was calculated as PE = (Colonies formed/Number of cells seeded) × 100%. Data are expressed as mean ± SEM, $n = 3$. The plating efficiencies were decreased in the WTMnSOD-overexpressing cells compared with the parental control cells (*$p = 0.0032$ for WT5 and *$p = 0.0012$ for WT6). The mutant MnSOD-overexpressing cells had increased plating efficiencies compared with the parental cells (**$p < 0.0001$ for clone H26L8 and **$p = 0.0462$ for clone H26L4).

DISCUSSION

The purpose of this work was to construct, express, and test a mutant MnSOD cDNA. We used site-directed mutagenesis to construct an active site mutant for MnSOD in which the histidine at amino acid 26 was replaced with a leucine. DNA sequencing demonstrated that we had produced the de-

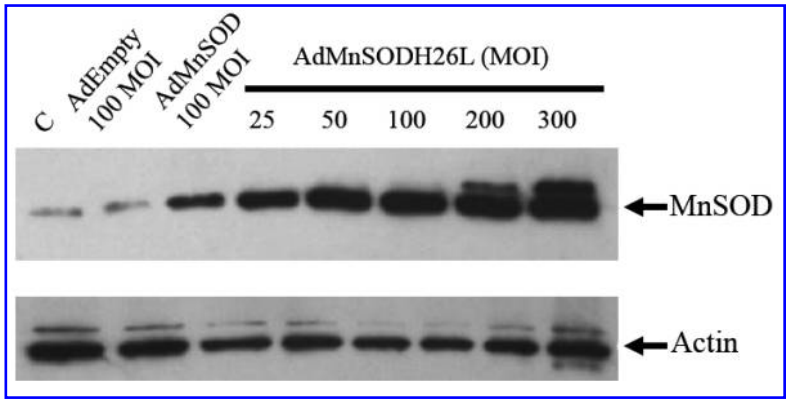

FIG. 6. Adenovirus MnSODH26L infection in MiaPaCa2 cells increased MnSOD protein in a dose-dependent manner. Cellular protein (10 μg) was separated on a 12.5% SDS polyacrylamide gel, and Western blotting was performed. The expression of MnSOD protein was observed in the ~22-kDa band. Actin was used as loading control.

sired mutation. We used three different transduction techniques to express the protein: transient plasmid transfection, stable plasmid transfection, and adenoviral transduction. All three techniques showed that we could overexpress both wild-type and mutant MnSOD protein. Western blotting, activity gel analysis, and spectrophotometric enzyme activity assay demonstrated that when we overexpressed wild-type MnSOD protein, we observed a large increase in both MnSOD immunoreactive protein and enzymatic activity. However, when we overexpressed the mutant MnSOD protein, we observed a large increase in MnSOD immunoreactive protein, but no increase in MnSOD enzymatic activity. These studies show that we have produced a mutant MnSOD protein with little or no enzymatic activity.

Not only did the mutant protein have little or no enzymatic activity, but it also did not produce the effects on cell function that the wild-type protein did. Thus, we found that wild-type protein reduced plating efficiency after plasmid transfection, whereas the mutant protein actually increased plating efficiency. Similarly, wild-type protein inhibited pancreatic cancer cell growth and reduced plating efficiency after adenovirus transduction, whereas mutant MnSOD inhibited growth

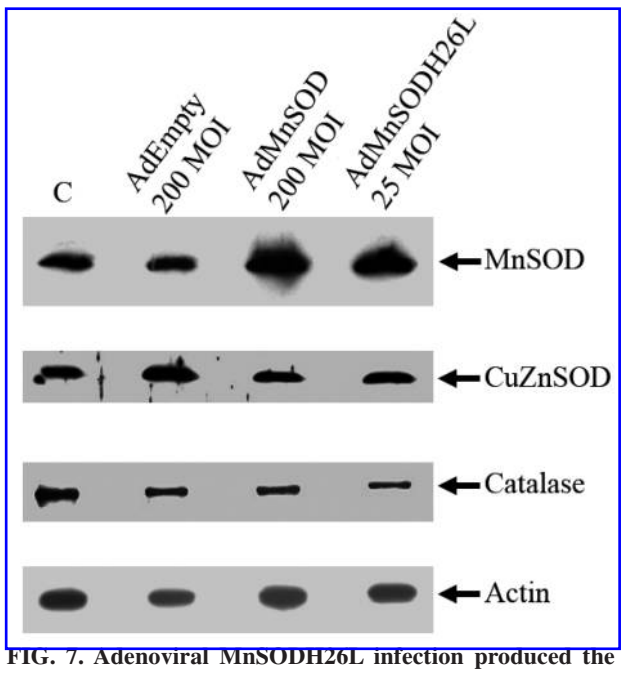

FIG. 7. Adenoviral MnSODH26L infection produced the same high MnSOD protein level as adenoviral wild-type MnSOD infection, but did not change CuZnSOD or catalase protein levels. Cellular protein (10 μg) was separated on a 12.5% SDS polyacrylamide gel, and Western blotting was performed. The expression of MnSOD, CuZnSOD, and catalase protein were observed in the ~22-, 16-, and 60-kDa bands, respectively. Actin was used as loading control. AdMnSODH26L-infected cells (25 MOI) had the same high MnSOD protein level as did AdWTMnSOD-infected cells (200 MOI), but did not change CuZnSOD or catalase protein levels.

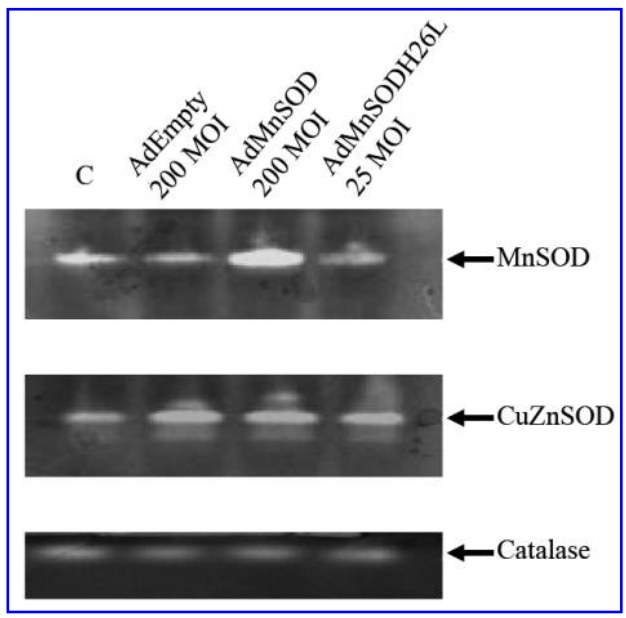

FIG. 8. Adenovirus MnSODH26L infection decreased MnSOD activity but did not change CuZnSOD or catalase activity. Cellular protein (10 μg) was separated in a 12% native polyacrylamide gel for SOD activity, and 30 μg of cellular protein was separated in an 8% native polyacrylamide gel for catalase activity. After electrophoresis, the gels were stained for SOD or catalase activity. Compared with the band from control (no treatment), and AdEmpty vector, the activity gel showed a greatly increased intensity of the activity band in the AdWTMnSOD-infected cells and decreased intensity of the activity band in the AdMnSODH26L-infected cells; however, the CuZnSOD or catalase activity did not change.

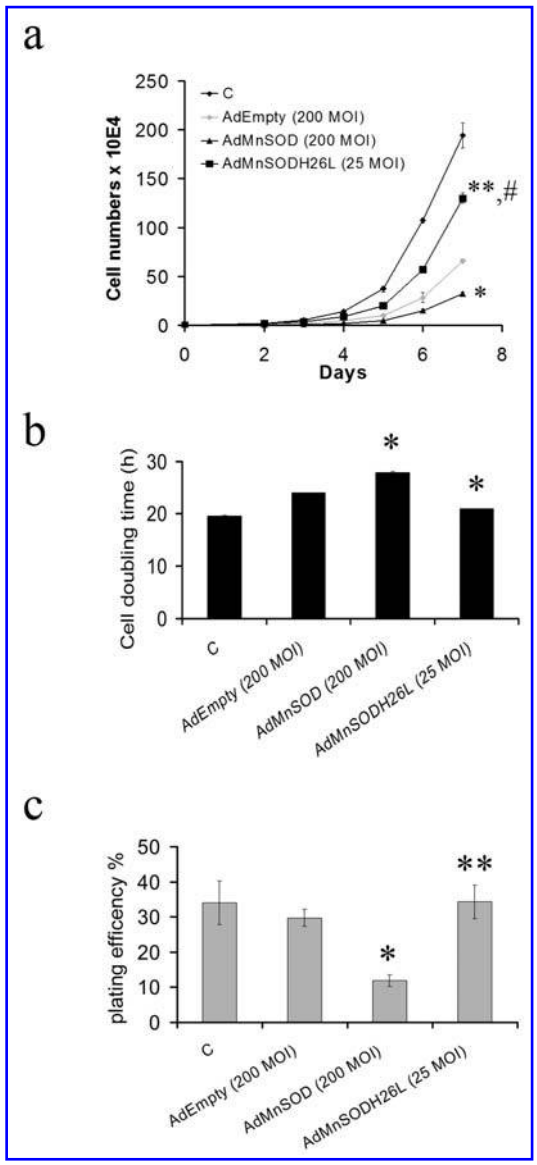

FIG. 9. Adenovirus MnSODH26L infection did not inhibit cell growth. (**a**) Growth curves: 5,000 cells were seeded into six-well plates and counted every 24 h by using a Coulter counter. The results represent mean ± SEM, $n = 3$. Adenoviral WTMnSOD (AdMnSOD in figure) inhibited cell growth compared with AdEmpty control cells ($*p < 0.05$). Conversely, AdMnSODH26L had a much smaller effect on cell growth and was statistically different from the cells treated with AdEmpty ($**p < 0.05$) or AdMnSOD ($\#p < 0.05$). (**b**) Cell doubling time. The doubling time was calculated from the growth curves during logarithmic growth phase. Data are expressed as mean ± SEM, $n = 3$. AdMnSOD-infected cells displayed a statistically significant increase in cell doubling time compared with AdEmpty-infected cells ($*p < 0.05$), whereas AdMnSODH26L-infected cells were significantly decreased ($*p < 0.05$) compared with AdEmpty-infected cells. (**c**) Plating efficiency. Cells were seeded into 60-mm dishes at a certain cell number. After 2 weeks incubation at 37°C, cells were fixed and stained. Colonies containing >50 cells were counted. The plating efficiency (PE) was calculated as PE = (Colonies formed/Number of cells seeded) × 100%. Data are expressed as mean ± SEM, $n = 3$. The plating efficiencies were decreased in the AdMnSOD cells compared with the parental cells and AdEmpty control cells (both $*p < 0.0001$). AdMnSODH26L increased plating efficiencies compared with the AdMnSOD ($**p < 0.0001$) and AdEmpty control cells ($p = 0.005$), but showed no difference compared with the parental cells ($p = 0.9855$).

to a much smaller extent and did not change plating efficiency. These results are consistent with what has been reported by Kim *et al.* (12) in a different cell system. In WI-38 VA13 subline 2RA SV40-transformed human lung fibroblasts, transduction with adenovirus containing wild-type MnSOD led to an increase in both MnSOD immunoreactive protein and enzymatic activity, whereas transduction with the same adenovirus used in the present study containing mutant H26L led to a large increase in MnSOD immunoreactive protein, but no increase in enzymatic activity. Moreover, Kim *et al*. showed very convincingly by using immunogold EM that the mutant protein localized in the mitochondria as expected. Thus, the mutant protein was found in the proper place in the cell but had little enzymatic activity. Finally, transduction with adenoviral wild-type MnSOD led to a large increase in the number of apoptotic cells, whereas transduction with the mutant MnSOD led to a much smaller increase in the number of apoptotic cells. Hence these authors also conclude, as we did, that the effects of MnSOD on cell function are largely due to the enzymatic activity of the protein.

Taken together, these studies suggest that the enzymatic activity of MnSOD is responsible for its effect on cell phenotype. This conclusion further suggests that either the species removed or produced by MnSOD causes its effects. We are currently investigating which of the species are responsible for the remarkable effects produced by this protein.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ANOVA, analysis of variance; BCS, bathocuproine disulfonic acid; BSA, bovine serum albumin; CAT, catalase; CuZnSOD, copper- and zinc-containing superoxide dismutase; DETAPAC, diethylenetriaminepentacetic acid; FBS, fetal bovine serum; HRP, horseradish peroxidase; LOH, loss of heterozygosity; MnSOD, manganese-containing superoxide dismutase; MOI, multiplicity of infectivity; NBT, nitroblue tetrazolium; PB, phosphate buffer; PBS, phosphate-buffered saline; PE, plating efficiency; ROS, reactive oxygen species; SOD, superoxide dismutase; Td, cell population doubling time; TEMED, *N,N,N′,N′*-tetramethyl-ethylenediamine; WT, wild type; XO, xanthine oxidase.

REFERENCES

1. Beauchamp C and Fridovich I. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Anal Biochem* 44**:** 276–287, 1971.
2. Beckman BS, Balin AK, and Allen RG. Superoxide dismutase induces differentiation of erythroleukemia cells. *J Cell Physiol* 139: 370–376, 1989.
3. Borgstahl GED, Parge HE, Hickey MJ, Beyer WF, Hallewell RA, and Tainer JA. The structure of human mi-

tochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* 71: 107–118, 1992.
4. Borgstahl GE, Parge HE, Hickey MJ, Johnson MJ, Boissinot M, Hallewell RA, Lepock JR, Cabelli DE, and Tainer JA. Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. *Biochemistry* 35: 4287–4297, 1996.
5. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding,. *Anal Biochem* 72: 248–254, 1976.
6. Bravard A, Sabatier L, Hoffschir F, Luccioni C, and Dutrillaux B. SOD2: a new type of tumor suppressor gene? *Int J Cancer* 51: 475–480, 1992.
7. Cerutti PA. Proxidant states and cancer. *Science* 227: 375–381, 1985.
8. Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson PE, Meltzer PS, and Trent JM. Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. *Proc Natl Acad Sci U S A* 90: 3113–3117, 1993.
9. Cullen JJ, Weydert C, Hinkhouse MM, Ritchie JM, Domann FE, Spitz DR, and Oberley LW. The role of manganese superoxide dismutase in the growth of pancreatic adenocarcinoma. *Cancer Res* 63:1297–1303, 2003.
10. Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen HV, Epstein CJ, and Huang T-T. CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 24: 367–380, 2005.
11. Fernandez-Pol JA, Hamilton PD, and Klos DJ. Correlation between the loss of the transformed phenotype and an increase in superoxide dismutase activity in a revertant subclone of sarcoma virus-infected mammalian cells. *Cancer Res* 42: 609–617, 1982.
12. Kim A, Oberley LW, and Oberley TD. Induction of apoptosis by adenovirus-mediated manganese superoxide dismutase overexpression in SV-40-transformed human fibroblasts. *Free Radic Biol Med* 39: 1128–1141, 2005.
13. Kondo T, Reaume AG, Huang TT, Murakami K, Carlson E, Chen S, Scott RW, Epstein CJ, and Chan PH. Edema formation exacerbates neurological and histological outcomes after focal cerebral ischemia in CuZn-superoxide dismutase knockout mutant mice. *Acta Neurochir Suppl* 70: 62–64, 1997.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227**:** 680–685, 1970.
15. Lam EWN, Hammad HM, Zwacka R, et al. Immunolocalization and adenoviral vector-mediated manganese superoxide dismutase gene transfer to experimental oral tumors. *J Dent Res* 79**:** 1410–1417, 2000.
16. Lam EWN, Zwacka R, Engelhardt JF, Davidson BL, Domann FE Jr, and Oberley LW. Adenovirus-mediated manganese superoxide dismutase gene transfer to hamster cheek pouch carcinoma cells. *Cancer Res* 57**:** 5550–5556, 1997.
17. Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, and Matsuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci U S A* 93: 9782–9787, 1996.
18. Li JJ, Oberley LW, St Clair DK, Ridnour LA, and Oberley TD. Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase. *Oncogene* 10: 1989–2000, 1995.
19. Li N, Oberley TD, Oberley LW, and Zhong W. Overexpression of manganese superoxide dismutase in DU145 human prostate carcinoma cells has multiple effects on cell phenotype. *Prostate* 35: 221–223, 1998.
20. Li Y, Huang T-T, Carlson EJ, Melov S, Ursell PC, Olson, JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, and Epstein CJ. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11: 376–381, 1995.
21. Liang BC, Ross DA, Greenburg HS, Meltzer PS, and Trent JM. Evidence for allelic imbalance of chromosome 6 in human astrocytomas. *Neurology* 44: 533–536, 1994.
22. Liu R, Oberley TD, and Oberley LW. Transfection and expression of MnSOD cDNA decreases tumor malignancy of human oral squamous carcinoma SCC-25 cells. *Human Gene Ther 8*: 585–595, 1997.
23. McCord JM, Keele BB, and Fridovich I. An enzyme based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc Natl Acad Sci U S A* 68: 1024–1027, 1971.
24. Melendez JA, Melathe RP, Rodriguez AM, Mazurkiewicz JE, and Davies KJA. Nitric oxide enhances the manganese superoxide-dependent suppression of proliferation in HT-1080 fibrosarcoma cells. *Cell Growth Differ* 10: 655–664, 1999.
25. Millikan D, Meese E, Vogelstein B, Witkowski C, and Trent J. Loss of heterozygosity for loci on the long arm of chromosome 6 in human malignant melanoma. *Cancer Res* 51: 5449–5453, 1991.
26. Oberley LW. Superoxide dismutase and cancer. In: Oberley LW, ed. *Superoxide Dismutase.* Vol II. Boca Raton, Fla: CRC Press, 1982.
27. Oberley LW and Buettner GR. Role of superoxide dismutase in cancer: a review. *Cancer Res* 39: 1141–1149, 1979.
28. Oberley LW and Oberley TD. Free radicals, cancer, and aging. In: Walford R, Harmon D, Miguel J, eds. *Free Radicals*, *Aging*, *and Degenerative Diseases*. New York: Alan R Liss, 1986:325–381.
29. Oberley LW and Oberley TD. Role of antioxidant enzymes in cell immortalization and transformation. *Mol Cell Biochem* 84: 147–153, 1988.
30. Oberley LW and Oberley TD. Reactive oxygen species in the aetiology of cancer. In: Ioannides C, Lewis DV, eds. *Drugs*, *Diet*, *and Disease.* Vol 1. New York: Prentice Hall, 1994:49–63.
31. Oberley TD and Oberley LW. Oxygen radicals and cancer. In: Yu BP, ed. *Free Radicals in Aging*. Boca Raton, Fla: CRC Press, 1993:248–267.
32. Oberley LW, Spitz DR. Assay of superoxide dismutase activity in tumor tissues. *Methods Enzymol* 105**:** 457–469, 1984.
33. Oberley LW, Spitz DR. Assay of superoxide dismutase using nitroblue tetrazolium. In: Greenwald RA, ed. *Hand-*

book of Methods for Oxy Radical Research. Boca Raton, Fla: CRC Press, 1985:217–225.
34. Ough M, Lewis A, Zhang YP, Hinkhouse MM, Ritchie JM, Oberley LW, and Cullen JJ. Inhibition of cell growth by overexpression of manganese superoxide dismutase (MnSOD) in human pancreatic carcinoma. *Free Radic Res* 38:1223–1233, 2004.
35. Safford SE, Oberley TD, Urano M, and St Clair DK. Suppression of fibrosarcoma metastasis by elevated expression of manganese superoxide dismutase. *Cancer Res* 54: 4261–4265, 1994.
36. Spitz DR, Elwell JH, Sun Y, Oberley LW, Oberley TD, Sullivan SJ, and Roberts RJ. Oxygen toxicity in control and H_2O_2-resistant Chinese hamster fibroblast cell lines. *Arch Biochem Biophys* 279**:** 249–260, 1990.
37. Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. *Anal Biochem* 179**:** 8–18, 1989.
38. St. Clair DK, Wan XS, Kuroda M, Vichitbandha S, Tsuchida E, and Urano M. Suppression of tumor metastasis by manganese superoxide dismutase is associated with reduced tumorigenicity and elevated fibronectin. *Oncol Rep* 4: 753–757, 1997.
39. St Clair DK, Wan XS, Oberley TD, Muse KE, and St Clair WH. Suppression of radiation-induced neoplastic transformation by overexpression of mitochondrial superoxide dismutase. *Mol Carcinog* 6: 238–242, 1992.
40. Sun Y, Elwell JH, Oberley LW. A simultaneous visualization of the antioxidant enzymes glutathione peroxidase and catalase on polyacrylamide gels. *Free Rad Res Commun* 5**:** 57–67, 1988.
41. Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang T-T, Nelson J, Strong R, and Richardson A. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* 16: 29–37, 2003.
42. Yan T, Oberley LW, Zhong W, and St. Clair DK. Manganese-containing superoxide dismutase overexpression causes phenotypic reversion in SV40-transformed human lung fibroblasts. *Cancer Res* 56: 2864–2871, 1996.
43. Zhang HJ, Yan T, Oberley TD, and Oberley LW. Comparison of effects of two polymorphic variants of manganese superoxide dismutase on human breast MCF-7 cancer cell phenotype. *Cancer Res* 59: 6276–6283, 1999.
44. Zhang HJ, Zhao W, Venkataraman S, et al. Metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. *J Biol Chem* 277**:** 20919–20926, 2002.
45. Zhong W, Oberley LW, Oberley TD, Yan T, Domann FE, and St. Clair DK. Inhibition of cell growth and sensitization to oxidative damage by overexpression of manganese superoxide dismutase in rat glioma cells. *Cell Growth Differ* 7: 1175–1186, 1996.
46. Zhong W, Oberley LW, Oberley TD, and St. Clair DK. Suppression of the malignant phenotype of human glioma cells by overexpression of manganese superoxide dismutase. *Oncogene* 14: 481–490, 1997.

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2. Ehab H. Sarsour, Maneesh G. Kumar, Amanda L. Kalen, Monali Goswami, Garry R. Buettner, Prabhat C. Goswami. 2011. MnSOD activity regulates hydroxytyrosol-induced extension of chronological lifespan. *AGE* . [CrossRef]
3. Ekambaram Ganapathy, Feng Su, David Meriwether, Asokan Devarajan, Victor Grijalva, Feng Gao, Arnab Chattopadhyay, G.M. Anantharamaiah, Mohamad Navab, Alan M. Fogelman, Srinivasa T. Reddy, Robin Farias-Eisner. 2011. D-4F, an apoA-I mimetic peptide, inhibits proliferation and tumorigenicity of epithelial ovarian cancer cells by upregulating the antioxidant enzyme MnSOD. *International Journal of Cancer* n/a-n/a. [CrossRef]
4. Aaron K. Holley, Sanjit Kumar Dhar, Yong Xu, Daret K. St. Clair. 2010. Manganese superoxide dismutase: beyond life and death. *Amino Acids* . [CrossRef]
5. Moo Rim Park, Feng Xu, Tao Cheng. 2009. The Effect of an Ectopic Overexpression of MnSOD in Mouse Hematopoietic Stem Cells. *The Korean Journal of Hematology* **44**:2, 82. [CrossRef]
6. Ehab H. Sarsour, Sujatha Venkataraman, Amanda L. Kalen, Larry W. Oberley, Prabhat C. Goswami. 2008. Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. *Aging Cell* **7**:3, 405-417. [CrossRef]
7. Tzu-Chao Chuang, Jah-Yao Liu, Chi-Tsai Lin, Ya-Ting Tang, Ming-Han Yeh, Su-Chien Chang, Jhy-Wei Li, Ming-Ching Kao. 2007. Human manganese superoxide dismutase suppresses HER2/neu-mediated breast cancer malignancy. *FEBS Letters* **581**:23, 4443-4449. [CrossRef]