Comparison of Effects of Two Polymorphic Variants of Manganese Superoxide Dismutase on Human Breast MCF-7 Cancer Cell Phenotype

Hannah J. Zhang, Tao Yan, Terry D. Oberley, and Larry W. Oberley

Radiation Research Laboratory, B180 Medical Laboratories, The University of Iowa, Iowa City, Iowa 52242 [H. J. Z., T. Y., L. W. O.]; Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital and Department of Pathology and Laboratory Medicine, The University of Wisconsin, Madison, Wisconsin 53705 [T. D. O.]

ABSTRACT

Two polymorphic variants of manganese superoxide dismutase (MnSOD), with either Ile or Thr at amino acid 58, (Ile$^{58}$MnSOD or Thr$^{58}$MnSOD), have been found in the human population. The MnSOD activity of these two variants and their effects on the malignant phenotype of human breast cancer MCF-7 cells were compared. It was demonstrated that MnSOD-overexpressing clones obtained from transfection of the two MnSOD cDNAs into MCF-7 cells had increased MnSOD immunoreactive protein and increased MnSOD activity. Cells overexpressing Ile$^{58}$MnSOD had 3-fold higher MnSOD activity than cells overexpressing Thr$^{58}$MnSOD in vivo at an equal MnSOD protein level. Tumor-suppressive effects of MnSOD-overexpressing cells were indicated by: (a) decreased plating efficiency; (b) elongated cell population doubling time; (c) lower clonogenic fraction in soft agar; and (d) complete inhibition or delayed onset of tumor formation in nude mice. When compared on the same activity basis, the suppressive effects of Ile$^{58}$MnSOD were similar to those of Thr$^{58}$MnSOD. However, far more Thr$^{58}$MnSOD protein was required to obtain the same amount of MnSOD activity, making the Thr$^{58}$MnSOD far less effective. A dose-response suppressive effect was observed when the increase of MnSOD activity was moderate. We conclude that MnSOD is a tumor suppressor in human breast cancer, but the Ile$^{58}$ form of the protein is a much less effective tumor suppressor than the Ile$^{58}$ form of the protein.

INTRODUCTION

ROS$^3$ are molecules that contain oxygen and have higher reactivity than ground-state molecular oxygen, examples being O$_2^\cdot$ and H$_2$O$_2$. In recent years, it has been proposed that ROS at low concentrations can activate signal transduction pathways and alter the expression of growth- and differentiation-related genes (1). However, ROS at high concentrations have detrimental effects on cells. Antioxidants are molecules that prevent or inhibit the oxidation process mediated by ROS. In addition to many small antioxidant molecules, there are many antioxidant proteins in vivo. SOD is one of the primary antioxidant enzymes. This family of enzymes catalyzes the dismutation of O$_2^\cdot$ to H$_2$O$_2$. Hydrogen peroxide is further detoxified to water by catalase and glutathione peroxidase. There are three isofoms of SOD in human cells: copper-zinc SOD, found predominantly in the cytoplasm; extracellular SOD; and MnSOD, which exists primarily in the mitochondrial matrix. Because in most cells mitochondria consume >95% of the cell’s oxygen, the mitochondrial electron transport chain is believed to be a principal source of endogenous ROS generation (2). MnSOD is the only known enzyme that scavenges O$_2^\cdot$ in the mitochondria. Decreased MnSOD levels may lead to increased ROS levels in mitochondria, thus increasing the susceptibility of cells to oxidative stress. ROS have been implicated in many diseases such as cancer (3), neurodegenerative disease (4), inflammation (5), ischemia-reperfusion injury (6), and diabetes (7), as well as aging (8). Therefore, MnSOD plays a pivotal role in protecting cells from ROS-induced oxidative damage.

Many previous studies have concluded that ROS related oxidative stress is an important factor in cancer formation (9). A consistently diminished amount of MnSOD has been observed in most types of primary cancers examined to date (10, 11). Many recent studies have focused on the tumor-suppressive effects of MnSOD (12–16). Similar results among these studies have indicated that increased levels of MnSOD suppressed the malignant phenotype as evidenced by slower cell growth rate, lower colony formation both in normal medium and in soft agar, and less tumor formation in nude mice when compared with the parental untransfected cells.

Very recently, it was shown that a polymorphism in human MnSOD may cause increased risk for breast cancer (17). A valine or alanine can be located at amino acid position 9 of the MnSOD presequence. Premenopausal women who were homozygous for the alanine allele had a 4-fold increase in breast cancer risk compared with those with one or two valine alleles. This suggests that MnSOD polymorphisms may be very important in cancer susceptibility. Mature human MnSOD is a homotrretameric enzyme consisting of 198 amino acids in each subunit. Another naturally occurring polymorphism has been found in the human population; either Ile or Thr can reside at the amino acid 58 position of MnSOD (18). In a pure enzyme study (19), the Thr$^{58}$MnSOD exhibited only half the enzymatic activity of the Ile$^{58}$MnSOD. It is believed that the loss of enzymatic activity is due to a packing defect at the tetrameric interface of the MnSOD enzyme caused by Thr$^{58}$.

A puzzle remained in transfection studies of MnSOD. In most of these studies, MnSOD-transfected cells had a much larger increase in MnSOD mRNA and protein levels than in MnSOD activity (15). It recently was realized that most of previous work was done using Thr$^{58}$MnSOD. Indeed, a previous study in our laboratory had shown a tumor-suppressive effect of Thr$^{58}$MnSOD on human breast cancer MCF-7 cells (13). No work has been done to compare the effects of transfection of the two MnSOD polymorphic variants on MnSOD activity and cancer cell phenotype. Therefore, our study goals were to compare the activity of two polymorphic variants of MnSOD in MCF-7 cells and to study the effects of these variants on the cancer cell phenotype. We transfected pcDNA3 plasmids containing either one of the two forms of the sense human MnSOD cDNA into MCF-7 cells and established 12 MnSOD-overexpressing clones. We here report that the cells overexpressing Ile$^{58}$MnSOD have 3-fold higher MnSOD activity than the cells overexpressing Thr$^{58}$MnSOD in vivo at an equal MnSOD protein level. We concluded that when the biological effects were compared on the basis of the same MnSOD activity, the tumor-suppressive effects of Ile$^{58}$MnSOD were similar to those of Thr$^{58}$MnSOD. Both polymorphic variants of MnSOD can suppress the tumor cell phenotype if the cells have the same MnSOD activity. However, because far more Thr$^{58}$MnSOD protein was required to

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2 To whom requests for reprints should be addressed, at Radiation Research Laboratory, B180 Medical Labs, College of Medicine, University of Iowa, Iowa City, IA 52242-1181.
3 The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; MnSOD, manganese superoxide dismutase; Thr$^{58}$MnSOD, Thr$^{58}$-containing MnSOD; Ile$^{58}$MnSOD, Ile$^{58}$-containing MnSOD; G418, geneticin; IDV, integrated density value; NBT, nitroblue tetrazolium; PE, plating efficiency; Td, cell population doubling time; WT, wild type.
achieve the same MnSOD activity, far more Thr\(^{58}\)MnSOD protein was needed to produce the same biological effects as Ile\(^{58}\)MnSOD. This makes Thr\(^{58}\)MnSOD less effective than Ile\(^{58}\)MnSOD as a tumor suppressor. Moreover, a dose-response suppressive effect was observed when the increase of MnSOD activity was moderate. These results suggested that expression of the Thr\(^{58}\) form of MnSOD in two alleles may also lead to increase in susceptibility to breast cancer.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells purchased from the American Type Culture Collection (Rockville, MD) were cultured in Eagle's MEM containing nonessential amino acids, 1 mSODce sodium pyruvate, and 10% fetal bovine serum. Medium was changed every 3–4 days, and cells were routinely incubated at 37°C in a humidified atmosphere with 5% CO\(_2\).

DNA Reconstruction and Site-directed Mutagenesis. Human MnSOD cDNA was a kind gift of Dr. Daret St. Clair of the University of Kentucky, Lexington, KY. It was sequenced by an automated fluorescent DNA sequencing analyzer, which revealed that this cDNA contained an ACO acodon for Thr at amino acid 58. Site-directed mutagenesis was performed to change ACA to ATA, the codon for Ile. The change was confirmed by DNA sequencing. The parental MCF-7 cell line was found to express the Ile form of MnSOD.

Transfection. MCF-7 cells were stably transfected with the pcDNA3 plasmids, containing either one of the two forms of the sense human MnSOD cDNA or containing no MnSOD insert, by the lipofectamine (Life Technologies) method. The G418-resistant colonies were isolated in the medium supplemented with 400 \(\mu\)g/ml G418 (Life Technologies). Five days before an analysis, cells were placed in culture medium without G418 supplement.

Protein Sample Preparation. The procedures for protein sample preparation were performed on ice. The cells were harvested by scraping. The cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.8), and sonicated with four bursts of 30 s each using a Vibra Cell Sonicator (Sonic and Materials Inc., Danbury, CT) with a cup horn at full power. Total protein concentrations were determined by the Bio-Rad (Hercules, CA) Bradford protein assay kit using bovine \(\gamma\)-globulin as standard.

Western Blot Analysis. A total of 10 \(\mu\)g of denatured protein was separated by 12.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and then probed with a specific rabbit antisera against human kidney MnSOD made in our laboratory (20). The MnSOD bands were detected by chemiluminescence ECL kit (Amersham, Arlington Heights, IL). The bands were examined with a computerized digital imaging system using Alphalmager 2000 software (Alpha Innotech, San Leandro, CA). The IDV was obtained by integrating all of the pixel values in the area of one band after correction for background.

Immunogold Staining. Cells were scraped from tissue culture dishes, fixed in Carson-Millonig’s fixative, and processed for immunogold immuno-histochemistry as described previously (21). Cells stained with anti-MnSOD antibody were photographed with a Philips electron microscope. The prints were then examined for the presence of immunogold beads. The immunogold beads had to be at least touching the outer mitochondrial membrane to be scored as mitochondrial labeling. As a control, normal rabbit serum was used in place of the primary antibody.

PCR. Total genomic DNA was isolated from 80% confluent cells using a DNAzol kit. PCR primers were selected according to the human MnSOD cDNA sequence (GenBank accession no. Y00985). The sequences of oligonucleotide primers were as follows: sense, 5'--CGGATCAGCGGTAGCAGCCA--3'; and antisense, 5'--CAGGATCACTTATACCACCTACA--3'. These primers were designed so that only one band of exogenous MnSOD gene was seen in MnSOD-transfected clones because the endogenous MnSOD gene (~8 kb) was too large to be synthesized under our PCR conditions. The PCR was performed as described previously (16). Twenty \(\mu\)l of PCR products were separated in a 1.5% agarose gel with ethidium bromide staining.

Northern Blot Analysis. Total RNA was extracted from 80% confluent cells using a RNAzol kit (Tel-test, Inc., Friendswood, TX). Northern blot analysis was performed as described previously (15). The membrane was hybridized with a 769-bp digoxigenin-labeled human MnSOD cDNA. SOD Activity Assays. MnSOD enzymatic activities were measured by the modified NBT method described by Spitz and Oberley (22) and a modified native activity gel assay as described by Beauchamp and Fridovich (23). MnSOD activity was quantified in the presence of 5 mM NaCN, which inhibits only the copper-zinc SOD activity. One unit of activity was defined as the concentration of SOD that inhibited the NBT reduction rate to half of the maximum.

Plating Efficiency and Cell Population Doubling Time. Cells (500–1000) were plated in 60-mm culture dishes, kept for 14 days to allow colony formation, and then stained with 0.1% crystal violet. The colonies containing \(\geq 50\) cells were scored. The plating efficiency (PE) was calculated as follows:

\[
PE = \frac{\text{Colonies formed/Cells seeded}}{\text{Cells seeded}} \times 100\%
\]

Twenty thousand cells of each clone were plated in a 24-well dish. The growth rate of cells was determined by counting the number of cells as a function of time. Cell population doubling time \((T_d)\) was calculated from the growth rate by the formula:

\[
T_d = \frac{0.693t\ln(N_i/N_f)}{t_i}
\]

where \(t\) is time in days, \(N_i\) is the cell number at time \(t\), and \(N_f\) is the cell number at the initial time.

Soft Agar Clonogenic Assay. Cells (1000–3000) were suspended in 3 ml of culture media with 0.3% agar. This was overlaid onto 3 ml of presolidified 0.5% agar in 60-mm dishes. After 4 weeks of incubation, the colonies larger than 0.1 mm in diameter were counted. The cell clonogenic fraction was calculated by:

\[
\text{Clonogenic fraction} = \frac{\text{Colonies counted}}{\text{Numbers of cells seeded}} \times 100\%
\]

Tumorigenicity in Nude Mice. Female nude (nu/nu) mice (4–5 weeks old; Harlan Sprague Dawley Co. Madison, WI) were used. Five million cells (0.1 ml) were s.c. injected into the back of each nude mouse. Four nude mice were used for each group. When a tumor was palpable, it was measured by a vernier caliper once every week for 20 weeks. Tumor volume \((TV)\) was calculated by the formula:

\[
TV (mm^3) = \left( L \times W^2 \right) / 2
\]

where \(L\) is the longest dimension of the tumor (in mm), and \(W\) is the shortest dimension of the tumor (in mm).

Statistics. ANOVA-Tukey’s multiple comparison was used to determine the statistical significance of the data at \(P = 0.05\). Pearson’s regression and correlation analysis was used to determine the relationship between the MnSOD activities and various parameters, including MnSOD protein levels, \(PE, T_d\), and clonogenic fraction.

Data Presentation. All of the data, except nude mouse results, were from the average of at least three independent experiments. In the cases of Western, Northern, and PCR experiments, representative results that were repeated at least three times with similar results are shown.

RESULTS

Nomenclature. Twelve clones with increased MnSOD protein levels were chosen: 8 clones from Ile\(^{58}\)MnSOD cDNA, and 4 clones from Thr\(^{58}\)MnSOD cDNA. The nomenclature for all clones is as follows: WT, parental MCF-7 cells; Neo4, a clone transfected with the pcDNA3 vector plasmid; SOD clones, clones from Thr\(^{58}\)MnSOD cDNA and designated SOD15, SOD18, SOD23, and SOD50; Mn clones, clones from Ile\(^{58}\)MnSOD cDNA and designated Mn1, Mn11, Mn28, Mn40, Mn44, Mn52, Mn59, and Mn63.

Verification of MnSOD Overexpression. The expression of MnSOD was verified by Western and Northern blot analyses, PCR, and immunogold immuno-histochemistry. The expression of MnSOD protein was measured by Western blot analysis. All 12 MnSOD trans-
fectants showed a large increase in MnSOD protein; the levels of MnSOD protein in WT and Neo4 were barely detectable (Fig. 1a). The Western band from SOD23 migrated at a higher position than the others. Sequencing analysis after PCR amplification of the inserted MnSOD cDNA revealed a point mutation at the stop codon, which led to a MnSOD protein in clone SOD23 that was 16 amino acids longer than that of WT. Likewise, Mn1 showed two almost equally intense bands, one at the expected position and the other at a much higher position. The reason for these changes are unclear. The intensity of the Western bands was measured. The resulting IDV was used to compare the amount of immunoreactive MnSOD protein. In the case of Mn1, only the band at the regular position was measured. All 12 MnSOD-transfected clones had a statistically significant increase in IDV compared with WT (Fig. 1d). The IDV of Neo4 was not statistically different from that of WT.

The expression of MnSOD mRNA was determined by Northern blot analysis. Fig. 1b shows that MnSOD-transfected clones expressed high levels of steady-state MnSOD mRNA. However, mRNA bands were not detectable in WT and Neo4 cells. The 1- and 4-kb MnSOD mRNA bands that usually are seen in many cell lines were not seen in these cells. This result suggested that the level of endogenous MnSOD mRNA in MCF-7 cells was very low. The fact that WT and Neo4 clones had nearly undetectable MnSOD protein levels supported this suggestion. PCR amplification of the genomic DNA also revealed that MnSOD cDNA was inserted into genomes of all MnSOD-transfected clones but not in WT and Neo4 (Fig. 1c). These results demonstrated that the foreign MnSOD cDNA has been stably transfected into the genome and expressed at the mRNA and protein levels.

To be a functional protein, MnSOD must be imported into mitochondria. Immunogold immunohistochemistry was thus performed to examine the location of MnSOD protein. The gold beads labeled mitochondria almost exclusively, with few gold beads observed in the cytoplasm or nucleus (Fig. 2a). MnSOD-transfected cells (Fig. 2, c, d, and f) showed an increase in specific mitochondrial labeling compared with WT (Fig. 2a) and Neo4 (Fig. 2b). Cells stained with normal rabbit serum did not show any mitochondrial labeling (Fig. 2e).

Increase of MnSOD Enzymatic Activity. The MnSOD enzymatic activity was measured by both NBT and activity gel assays. Fig. 3a shows the activity gel result, where the intensity of an activity gel band corresponds to the MnSOD activity of that clone. All transfected clones showed increased MnSOD activity with the possible exceptions of clones SOD18 and SOD23. The activities of the Mn group was higher than that of the SOD group. Clones Mn11 and Mn40 had the highest MnSOD activity. Clones SOD15 and SOD50 showed two bands in the activity gel. We believe that the lower bands were from exogenous Thr58MnSOD; Thr58MnSOD is expected to migrate faster than Ile58MnSOD in a native gel system because of the change from a less negatively charged Ile to a more negatively charged Thr. In addition, the upper bands appeared to be from endogenous MnSOD because they had the same migration and brightness as the WT. These observations demonstrated that both forms of MnSOD have enzymatic activity. The lower band was not observed in SOD18, probably because the exogenous MnSOD activity was too low. The SOD23 band migrated slightly higher than the others and did not show an endogenous band. All Mn clones had only one band at the endogenous MnSOD position.

The MnSOD enzymatic activity was measured by the NBT assay. As shown in Fig. 3b, 11 of 12 MnSOD-transfected clones had statistically significant increases in MnSOD activity compared with WT, and the activity of Neo4 was not different from that of WT. Similar to the activity gel result, Mn11 and Mn40 had the highest activities. SOD15 and SOD50 had higher MnSOD activities than SOD23 and SOD18.

Comparison of MnSOD Activity between Ile58MnSOD and Thr58MnSOD. To further compare the differences of MnSOD activity between Ile58MnSOD and Thr58MnSOD, the relationships between the MnSOD protein level and MnSOD activity for the Mn and SOD groups were studied. The relative IDV and the relative MnSOD activity expressed as fold increase over WT were calculated as shown in Table 1 and plotted in Fig. 4. The regression analysis yielded two straight lines with two different slopes: 0.9 for the Mn group and 0.3 for the SOD group. Both lines had positive linear relationships with correlation coefficients of $r = 0.942$ and 0.970, respectively. These relationships demonstrated that increases in the MnSOD protein levels are strongly related to increases in the enzymatic activity in MnSOD-transfected cells. However, the two different slopes indicated different proportional increases in the MnSOD activity compared with the MnSOD protein levels between the two groups. The ratio of two slopes was 3:1 (Mn group:SOD group), which demonstrated that the Ile58MnSOD had 3-fold higher specific MnSOD activity than the Thr58MnSOD, when values were adjusted for the same amount of MnSOD protein.

Fig. 1. Verification of MnSOD overexpression at protein, mRNA, and genomic levels. a, Western blotting analysis demonstrated an increase in MnSOD at the protein level in all MnSOD-transfected clones. b, Northern blotting analysis showed that one band existed in all MnSOD-transfected clones. c, PCR confirmed that the MnSOD cDNA had inserted into cellular genomic DNA. d, densiometric analysis of Western blotting bands showed increases in MnSOD protein levels from all MnSOD-transfected clones. The mean IDV ± SD from four independent Western blots of different samples is shown. * $P < 0.05$ compared with WT. WT, parental MCF-7 cells; Neo4, vector control clone; SODs, cells transfected with Thr58MnSOD; Mns, cells transfected with Ile58MnSOD; +, positive control for PCR using MnSOD cDNA as template; –, PCR reaction without DNA template as negative control. Bars, SD.
Effects of Two Forms of MnSOD on MCF-7 Cell Growth in Vitro and in Vivo. Tumor cell growth characteristics were used to evaluate the effects of the overexpression of both forms of MnSOD in this system.

Td was measured as shown in Fig. 5a. Compared with WT and Neo4, which had Tds of 2.1 and 2.0 days, MnSOD-transfected cells displayed statistically significant increases in Td, ranging from 2.4 days for SOD18 to 3.2 days for Mn52. The average Td from the SOD group was also compared with the average Td of the Mn group. The average Td (2.6 ± 0.2 days) for the SOD group was not statistically different from the average Td (2.7 ± 0.3 days) for the Mn group. Instead, as shown in Fig. 5b, the elongation of Td was correlated with the increase of MnSOD activity up to 44 units/mg protein ($r = 0.87; P < 0.05$). However, this correlation was not present when MnSOD activity was >44 units/mg protein (an ~6-fold increase in activity).

The ability of the cells to form colonies was measured by PE (Fig. 6a). Compared with Neo4, all Mn clones except Mn63 showed statistically significant decreases in PE, ranging from 4% for Mn52 to 16% for Mn28. Mn63 had 20% PE, which was lower than Neo4 but higher than WT. SOD15 and SOD50 had statistically significant less colony formation (8 and 14%) compared with Neo4. SOD18 and SOD23 (21% for both) were not significantly different from Neo4. The PE for the SOD group versus the Mn group was also compared. An average PE of 13 ± 6% for the Mn group was found to be no
different from the average PE of 16 ± 5% for the SOD group. Instead, a strong correlation between the decrease in PE and the increase in MnSOD activity was found (r = −0.74; P < 0.05) when MnSOD activity was <44 units/mg protein (Fig. 6b). However, when MnSOD activity was >44 units/mg protein, the correlation was no longer present.

Cell anchorage-dependent growth was measured by soft agar assay. Cell growth ability in soft agar is believed to be an in vitro indicator of the in vivo malignant phenotype. Compared with WT and Neo4 with 3.90 and 3.60% clonogenic rates in soft agar, all clones in the Mn group had statistically significant decreases in clonogenic ability in soft agar, ranging from 0.69% for Mn52 to 2.43% for both Mn40 and Mn11 (Fig. 7a). The clonogenic fractions for SOD15 (0.12%) and SOD50 (0.43%) were also statistically lower than those for WT and Neo4. SOD23 and SOD23 each had only 1.5- and 1.3-fold increases in MnSOD activity, and these increases were apparently not sufficient to inhibit tumor formation. A total of 62.5% of the mice in these two low-expressing clones formed tumors. These tumors grew at the same rate as those of WT and Neo4. All of the tumors were removed from the mice at the end of the 20-week period. The levels of MnSOD in these tumors were examined by Western blot analysis. All of the tumors maintained MnSOD levels similar to those in the cells that had been injected (data not shown). This observation suggested that the tumors we measured originated from the MnSOD-transfected MCF-7 cells and did not spontaneously arise within the mice, because spontaneous tumors would have had low MnSOD activity.

**DISCUSSION**

Although it has been shown that purified Thr58MnSOD protein had half the activity of the Ile58MnSOD protein in the test tube, here for the first time, we report that Thr58MnSOD had only one-third of the Ile58MnSOD activity in a living cell system. We believe that this finding has important clinical relevance because Thr58MnSOD occurs naturally in the human population as a polymorphic variant. The association of MnSOD defects with many diseases has attracted increasing attention in recent years. For example, MnSOD with de-

**Table 1** Relative fold increases of MnSOD protein level and MnSOD activity

<table>
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<th>Cell line</th>
<th>Relative IDV (fold)</th>
<th>Relative activity (fold)</th>
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<td>Mn63</td>
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The most striking result was from the in vivo tumorigenicity study in nude mice. This experiment was designed to test the ability of MnSOD-transfected cells to form tumors in vivo. During a 20-week period, 85% of mice injected with WT or Neo4 cells developed tumors. In contrast, only 15.6% of mice in the Mn group formed tumors (Table 2), and these tumors grew much slower than those of WT and Neo4 (Fig. 8). Three of the five tumors formed in the Mn group had delayed onset of tumor formation. Although 7 of 16 mice (43.75%) in the SOD group grew tumors, in clones SOD15 and SOD50, with a 3-fold increase in MnSOD activity, only one mouse from each clone had a tumor (25%). These tumors also grew slower than those of WT and Neo4 and did not appear until week 10. SOD18 and SOD23 each had only 1.5- and 1.3-fold increases in MnSOD activity, and these increases were apparently not sufficient to inhibit tumor formation. A total of 62.5% of the mice in these two low-expressing clones formed tumors. These tumors grew at the same rate as those of WT and Neo4. All of the tumors were removed from the mice at the end of the 20-week period. The levels of MnSOD in these tumors were examined by Western blot analysis. All of the tumors maintained MnSOD levels similar to those in the cells that had been injected (data not shown). This observation suggested that the tumors we measured originated from the MnSOD-transfected MCF-7 cells and did not spontaneously arise within the mice, because spontaneous tumors would have had low MnSOD activity.
creased activity has been found in leukocytes of diabetics (7). SOD protected healthy pancreatic islet tissue after transplantation into diabetic animals (24). In addition, a diallelic polymorphism (from valine to alanine) in the mitochondrial target sequence of MnSOD has been found in the Japanese population (25). This polymorphic variant, which was believed to influence its mitochondrial import ability, has been associated with Parkinson’s disease. Moreover, it recently has been reported that premenopausal women who are homozygous for the alanine allele have a 4-fold increase in breast cancer risk compared with those with one or two valine alleles (17). Decreased levels of MnSOD consistently have been seen in cancer cells (26). Furthermore, mitochondrial DNA is particularly prone to oxidative damage because of a lack of histone protection and poor DNA repair ability. Miquel et al. (27, 28) have proposed that oxidative damage to mitochondrial DNA would lead to physiological aging. This mitochondrial DNA mutation theory of aging presently is undergoing intense scrutiny. Therefore, humans expressing Thr58 MnSOD may have the disadvantage of lower MnSOD protection against long-term oxidative damage and thus may be prone to many of these diseases. Early detection of this form of MnSOD by molecular methods such as genomic screening may someday help prevent or delay the onset of such diseases.

Three criteria for measuring cell growth are generally recommended to determine the characteristics of the tumor cell malignant phenotype: (a) in vitro cell growth measured by growth rate and clonogenic ability; (b) in vitro cell survival in soft agar; and (c) in vivo tumor formation in nude mice. It is generally believed that in vitro tumor cells have the ability to grow faster, to form more colonies, and to survive better in soft agar than normal cells. In vivo, tumor cells can form tumors in immunodeficient mice. We evaluated our MnSOD-overexpressing cells by these three criteria for tumor cell malignant phenotype. Our results demonstrated that the increase of MnSOD activity in human breast cancer MCF-7 cells inhibited cell growth rate with elongated Tds in most of MnSOD-overexpressing clones. These MnSOD-overexpressing cells also showed lower PE, lower clonogenic fraction in soft agar, and delayed onset or no tumor formation in nude mice than their counterparts, the parental nontransfected cells and vector control cells. Previously, a generally lower MnSOD activity was observed in many types of tumors when compared with the normal cell type from which the tumor arose (29–32). A number of studies have also suggested an inverse correlation between MnSOD levels and malignancy (21, 33, 34). Therefore, MnSOD had been hypothesized as a tumor suppressor (35). Our results suggested that MnSOD is a tumor suppressor in human breast cancer.

In addition, we also compared the effects of two MnSOD polymor-
phic variants on the MCF-7 cancer cell phenotype based on the three criteria for tumor cell phenotype. We found, in general, both polymorphic variants of MnSOD inhibited tumor cell growth and phenotype despite the difference in MnSOD activity in vivo. Ile58MnSOD with 3-fold higher MnSOD activity than Thr58MnSOD showed similar effects in inhibiting the tumor cell phenotype when the comparison was based on an equal enzymatic activity. However, it required a much larger amount of Thr58MnSOD protein to produce the same enzymatic MnSOD activity and biological effects as Ile58 MnSOD. One effect of note was that transfection with the Ile58 MnSOD led to much higher levels of MnSOD: not only was there more activity per protein, there was also much more MnSOD protein in some of the clones. This suggested that Thr58 MnSOD may lead to lower MnSOD activity for a variety of reasons.

Furthermore, we studied the correlation between MnSOD activity and the parameters for measuring tumor cell malignancy. We found that at certain level, when the MnSOD activity was moderate, the inhibition effects of MnSOD on tumor cells correlated well with its activity. In our cell model, a MnSOD activity of 44 units/mg protein seemed to be the limit at which MnSOD suppressive effects reached the maximum. Below this level, the increase in MnSOD activity was proportionally related to the suppression of MCF-7 cell growth measured by cell PE, Td, and cell clonogenic fraction in soft agar. Above this level, the suppression effects of MnSOD existed but did not correlate with the MnSOD activity. This suggests that MnSOD expression exceeding 44 units/mg protein can lead to detrimental effects. Indeed, Mn40, the highest MnSOD-overexpressing clone, had the highest tumor incidence among the Mn clones. The reason for this detrimental effect is under active investigation in our laboratory.

**REFERENCES**

EFFECTS OF MnSOD VARIANTS


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