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Inhibition of Cell Growth by Overexpression of Manganese Superoxide Dismutase (MnSOD) in Human Pancreatic Carcinoma

MATTHEW OUGH[a], ANNE LEWIS[a], YUPING ZHANG[b], MARILYN M. HINKHOUSE[c], JUSTINE M. RITCHIE[d], LARRY W. OBERLEY[b,d] and JOSEPH J. CULLEN[b,c,d,e,*]

[a]*University of Iowa College of Medicine, Iowa City, IA, USA;* [b]*Radiation Oncology, Iowa City, IA, USA;* [c]*Veterans Affairs Medical Center, Iowa City, IA, USA;* [d]*The Holden Comprehensive Cancer Center, Iowa City, IA, USA;* [e]*Department of Surgery, Iowa City, IA, USA*

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Manganese superoxide dismutase (MnSOD) levels have been found to be low in human pancreatic cancer [*Pancreas* **26**, (2003), 23] and human pancreatic cancer cell lines [*Cancer Res.* **63**, (2003), 1297] when compared to normal human pancreas. We hypothesized that stable overexpression of pancreatic cancer cells with MnSOD cDNA would alter the malignant phenotype. MIA PaCa-2 cells were stably transfected with a pcDNA3 plasmid containing sense human MnSOD cDNA or containing no MnSOD insert by using the lipofectAMINE method. G418-resistant colonies were isolated, grown and maintained. Overexpression of MnSOD was confirmed in two selected clones with a 2–4-fold increase in MnSOD immunoreactive protein. Compared with the parental and neo control cells, the MnSOD-overexpressing clones had decreased growth rates, growth in soft agar and plating efficiency *in vitro*, while *in vivo*, the MnSOD-overexpressing clones had slower growth in nude mice. These results suggest that MnSOD may be a tumor suppressor gene in human pancreatic cancer.

Keywords: Dicumarol; Radiation; Reactive oxygen species; Manganese superoxide dismutase; Pancreatic cancer

Cells contain a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species (ROS). There are three major types of primary intracellular antioxidant enzymes in mammalian cells—superoxide dismutase (SOD), catalase and peroxidase, of which glutathione peroxidase (GPx) is the most prominent. The SODs convert $O_2^{\cdot-}$ into H_2O_2, while the catalases and peroxidases convert H_2O_2 into water. In this way, two toxic species, $O_2^{\cdot-}$ and H_2O_2, are converted to the harmless product water. These antioxidant enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells.[1] An important feature of these enzymes is that they are highly compartmentalized. In general, manganese containing superoxide dismutase (MnSOD) is localized in the mitochondria, copper- and zinc-containing superoxide dismutase (CuZnSOD) in the cytoplasm, catalase in peroxisomes and cytoplasm and GPx in many subcellular compartments. Each of these enzymes is also found in several isoforms. One reason for the existence of many forms of each of these enzymes is to reduce oxidative stress in the various parts of the cell; different proteins are needed for different cellular and subcellular locations.

As with most other solid tumors, pancreatic cancer has been demonstrated to have low levels of antioxidant enzymes.[2] Immunohistochemistry demonstrated that MnSOD, CuZnSOD, catalase and GPx protein are decreased in human pancreatic ductal carcinoma specimens when compared to normal human pancreas. Similar findings are seen in primary human pancreatic cancer cell lines, including the pancreatic cancer cell line MIA PaCa-2, which has decreased levels of MnSOD immunoreactivity and enzyme activity when compared to normal human pancreas.[3] This study also demonstrated

*Corresponding author. Address: 4605 JCP, University of Iowa Hospitals and Clinics, Iowa City, IA 52242, USA. Tel.: +1-319-353-8297/339-5497. Fax: +1-319-356-8378. E-mail: joseph-cullen@uiowa.edu

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that cell-doubling time was most rapid in the cell lines with the lowest levels of MnSOD. There was no correlation between cell growth and the levels of other antioxidant enzymes including CuZnSOD, catalase or GPx; however, MnSOD activity and immunoreactive protein correlated with pancreatic tumor cell doubling time. These findings suggest that MnSOD may play a role in the growth of pancreatic cancer *in vitro*.

Further confirmation that MnSOD may play a role in the growth of pancreatic cancer was provided in another study from our laboratory, MnSOD was overexpressed in pancreatic cancer cells by infection with an adenovirus-MnSOD construct.[4] Cell growth, plating efficiency and growth in soft agar decreased with increasing amounts of the adenovirus MnSOD construct. These results further suggest that MnSOD may be a tumor suppressor gene in human pancreatic cancer.

Although, MnSOD has been stably overexpressed in several tumor cell types, the rationale for doing yet another tumor type is 4-fold. Firstly, many of the known oncogenes and tumor suppressor genes are cell-type specific. We wanted to determine if pancreatic cancer is responsive to MnSOD overexpression. Secondly, we wanted to determine if pancreatic cancer cells are responsive to MnSOD overexpression utilizing a different technique than adenovirus infection, specifically liposomal-mediated plasmid transfection. Adenovirus transduction can have non-specific effects unrelated to the gene being transduced. Thirdly, pancreatic cancer is now the fourth leading cause of cancer death in the United States with an overall 5-year survival rate of less than 5%.[5] Even after curative resection, the 5-year survival rates achieved at specialized centers are less than 20% and the majority of patients die of metastatic cancer recurrence.[6] Thus, novel treatment strategies directed against this devastating malignancy are greatly needed, which leads to the final reason for performing these studies. Many have suggested that the mechanism of MnSOD overexpression is due to increased H_2O_2 levels.[7,8] If $O_2^{\cdot-}$ could be generated in greater amounts in the presence of MnSOD overexpression, then theoretically, more cell killing would occur when $O_2^{\cdot-}$ dismutes to H_2O_2. To study this final rationale, we treated wild-type, vector alone and MnSOD overexpressing clones with two modalities that increase intracellular production of $O_2^{\cdot-}$, ionizing radiation[9] and dicumarol.[10]

MATERIALS AND METHODS

Cell Culture and Transfection

MIA PaCa-2 cells were purchased from American Type Culture Collection (Manassas, VA) and are human primary pancreatic adenocarcinoma cells derived from tumor tissue of the pancreas obtained from a 65-year old male. The cell cultures were maintained at 37°C in Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and 2.5% horse serum and were negative for Mycoplasma. The cells at passage 40 were transfected with the pcDNA3 plasmid containing a sense human MnSOD cDNA that was cloned into the Kpn1 and EcoR1 site and controlled by the CMV promoter.[11] The selection marker control was rendered by transfection with only the pcDNA3vector. The transfection was performed with Lipfect-AMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's recommendations. The clones were selected with 500 μg/ml of G418 (Geneticin; Life Technologies, Inc.) for 2 weeks after the transfection and screened by Western blotting for the level of MnSOD expression.

Cell Homogenization and Protein Determination

Cells were washed three times in PBS (pH 7.0), scraped from the dishes using a rubber policeman and then collected in potassium phosphate buffer (pH 7.8). This was followed by sonic disruption on ice for 30 s in 10-s bursts using a VibraCell sonicator (Sonics and Materials Inc., Danbury, CT) at 100% power. Protein concentration was determined using the Bio-Rad Bradford dye binding protein assay kit (Hercules, CA) according to the manufacturer's instructions.

Western Analysis

Immunoreactive protein corresponding to MnSOD was identified and quantitated from total cell protein by the specific reaction of the immobilized protein with its antibody. Total protein was electrophoresed in a 12.5% SDS-polyacrylamide running gel and a 5% stacking gel. Gels were stained for protein loading with Coomassie blue staining to determine equal loading. The proteins were then electotransferred to nitrocellulose sheets. After blocking in 20% fetal bovine serum for 1 h, the sheets were washed and then treated with antisera to MnSOD (1:1000) for 1 h. Polyclonal rabbit-anti-human antibodies to MnSOD has been prepared and characterized in our laboratory.[12] This antibody has been shown to react with the appropriate protein in a variety of species, including hamster and human.[11,12] The blot was incubated with horseradish peroxidase-conjugated goat-anti-rabbit (Sigma) IgG (1:10,000) for 1 h at room temperature. The washed blot was then treated with ECL western blot detection solution (Amersham Life Science, Buckinghamshire, United Kingdom) and exposed to X-ray film. Western blots were performed in duplicate.

SOD Activity Assay

SOD activity was measured using an indirect competition assay between SOD and an indicator molecule, nitroblue tetrazolium. This was performed in the crude homogenate according to the method of Spitz and Oberley.[13] Sodium cyanide (5 mM) inhibits Cu/ZnSOD; therefore, activity measured in the crude homogenate in the presence of sodium cyanide indicates only MnSOD activity. Specific activity was reported as units per mg protein.

GPx Activity

Aliquots of sample were assayed according to an established procedure[14] in potassium phosphate buffer, pH 7, containing glutathione, glutathione reductase, NaN_3 to inhibit catalase and NADPH. Hydrogen peroxide was used as the substrate.

Catalase Activity

Catalase activity was measured by the method of Beers and Sizer[15] with the analysis of Aebi.[16] All measurements were normalized to protein content. Briefly, this is a spectrophotometric procedure that measures peroxide removal.

Antioxidant Enzyme Activity Gels

In this technique, non-dissociating slab gels were run essentially by the method of Davis[17] with ammonium persulfate used as the initiator in the running gel (12.5%) and riboflavin-light in the stacking gel (5%). Once run, the gels were stained for SOD activity by the method of Beauchamp and Fridovich.[18] Cu/ZnSOD and MnSOD were differentiated by the presence of sodium cyanide in the staining solution, which inhibits Cu/ZnSOD. The protein concentration was measured by the method of Lowry.[19] All antioxidant enzyme activity gels were performed in duplicate.

Cell Growth

Cells (1×10^4) were plated in triplicate in 1.5 ml complete media in 24-well plates. Cells were trypsinized and then counted on alternate days for 2 weeks using a hemocytometer. Cell population doubling time in hours (DT) was determined using the following equation:

$$\mathrm{DT\,(hours)} = 0.693(t - t_0)/\ln(N_t/N_0)$$

where t_0 is the time at which exponential growth began, t the time in hours, N_t the cell number at time t and N_0 is the initial cell number.[4]

Plating Efficiency

Wild type, neo, Mn1 and Mn7 clones (5×10^3) were plated in triplicate into 60-mm dishes in complete media. The dishes were maintained in the incubator for 6 days to allow colony formation. The colonies were then fixed and stained with 0.1% crystal violet and 2.1% citric acid and those colonies containing greater than 50 cells were scored.

Anchorage-independent Growth in Soft Agar

Wild type, neo, Mn1 and Mn7 clones (5×10^3) were suspended in 3 ml of complete media containing a solution of 6% agar in double distilled H_2O so that the final concentration of the agar was 0.3%. This suspension was then plated over 3 ml of complete media made using a 6% agar solution in double distilled H_2O so that the final concentration of the bottom agar was 0.5%. After 16 days, colonies of greater than 0.1 mm in diameter were scored. The clonogenic fraction was determined using the following equation:

$$\text{Soft Agar Plating Efficiency (PE)} = (\text{colonies formed/cells seeded}) \times 100.$$

MTT Assay

Cells (5×10^3) were seeded in 96-well plates either in 50 μl of medium overnight followed by addition of dicumarol diluted in 50 μl of medium or in 100 μl of medium overnight followed by exposure to γ radiation (1.5 Gy/min) from a ^{137}Cs source, and the cells were allowed to grow for 3 days. Ten microliter of 5 mg/ml MTT were added to the cultures for 3 h incubation followed by the addition of 100 μl of lysing buffer containing 20% SDS and 50% *N*,*N*-dimethyl formamide, pH 4.7 at 37°C overnight. Color development was measured by a microplate titer reader (Bio-Rad) at 590 nm.

Clonogenic Survival

To determine the effect of MnSOD-overexpression combined with different modalities that increase intracellular production of $O_2^{\cdot -}$, cells were treated with dicumarol (0, 50 and 100 μM) for 4 and 24 h or γ radiation from a ^{137}Cs source at doses of 0, 2, 4 and 6 Gy. The cells were then plated for 7 days to form colonies. Colonies were stained and counted as described under plating efficiency. Cellular surviving fraction was calculated as follows:

$$\text{Surviving fraction} = \text{colonies formed/cells seeded/PE} \times 100.$$

Nude Mice

Thirty-day-old athymic nude mice were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The nude mice protocol was reviewed and approved

by the Animal Care and Use Committee of the University of Iowa on July 5, 2001. The animals were housed four to a cage and fed a sterile commercial stock diet and tap water, *ad libitum*. Animals were allowed to acclimate in the unit for 1 week before any manipulations were performed. Each experimental group consisted of four mice. Wild type (MIA PaCa-2), neo and the MnSOD-overexpressing clones (2×10^6) were delivered subcutaneously into the flank region of nude mice from a 1-cm^3 tuberculin syringe equipped with a 25-gauge needle. Tumor size was measured weekly by means of a vernier caliper and tumor volume was estimated according to the following formula: tumor volume $= (\pi/6) \times L \times W^2$, where L is the greatest dimension of the tumor and W is the dimension of the tumor in the perpendicular direction.[4] Animals were killed by CO_2 asphyxiation when the tumors reached a predetermined size of 10×10 mm and this was considered the time to sacrifice.

Statistical Analysis

Statistical analysis for the *in vitro* studies was performed using SYSTAT. A single factor ANOVA, followed by *post-hoc* Tukey test, was used to determine statistical differences between means. For non-parametric data, a Wilcoxon rank sum test was used. All means were calculated from three different cultures, and error bars represent standard error of mean (SEM). All western blots, activity assays and activity gel assays were repeated at least twice.

To compare the treatment groups over time for tumor volume, the linear mixed model analysis[20] assuming either an auto-regressive order 1 or compound symmetry covariance structure for within subjects was used. Selection of the covariance structure was based on the Akaike's Information Criterion.[21] In the linear mixed model analysis, group was considered a fixed effect and day was considered a continuous. For the *in vivo* studies, tumor volume was compared among the four groups using the data from days 1 through 28. Survival curves were estimated by the Kaplan–Meier product limit curves. The log rank test was used to compare survival between the groups. All data analyses for the *in vivo* data were done using SAS version 8.0. Western blots and activity gels were done at least twice.

RESULTS

Transfection of Human MnSOD cDNA into MIA PaCa-2 Cells

Western Blotting and Activity Gels

MIA PaCa-2 cells were transfected with the pcDNA3 plasmid containing a sense human MnSOD cDNA or pcDNA3 plasmid alone as a control. Two MnSOD-overexpressing cell lines and one neo-vector control cell lines was obtained under G418 selection. Western blot analysis was used to measure the amount of immunoreactive MnSOD protein in the MIA PaCa-2 cell lines (Fig. 1A). The parental cell line and neo

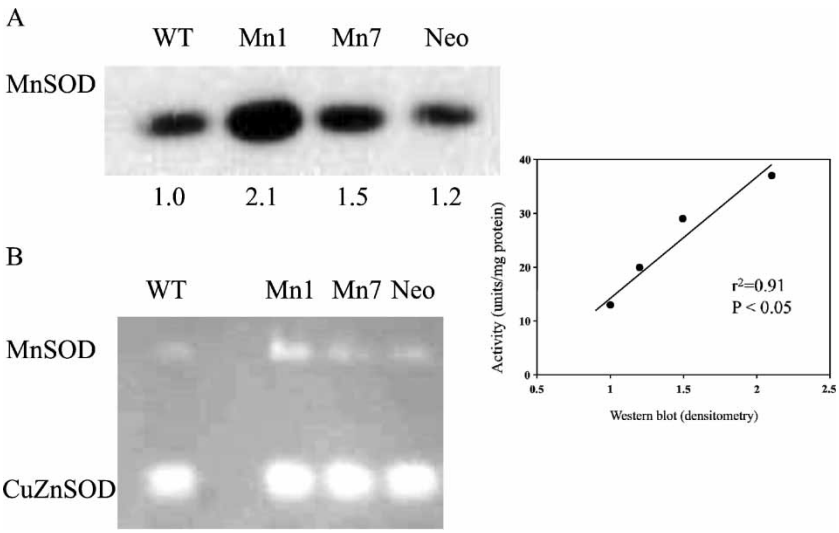

FIGURE 1 (A) Western blotting analysis for MnSOD. Total protein was electrophoresed in a 12.5% SDS-polyacrylamide running gel and a 5% stacking gel. After blocking in 20% fetal bovine serum for 1 h, the sheets were washed and then treated with polyclonal rabbit-anti-human antibodies to MnSOD. MIA PaCa-2 cells were transfected with the pcDNA3 plasmid containing a sense human MnSOD cDNA that was cloned into the Kpn1 and EcoR1 site and controlled by the human CMV promoter. The transfection was performed with LipfectAMINE. All western blots were repeated to confirm the findings. Linear regression analysis indicated a positive correlation of MnSOD immunoreactivity vs. MnSOD activity. (B) Detection of MnSOD activity by the activity gel assay in the parental wild-type, neo control (V2b), Mn1 and Mn7 clones. Increases in MnSOD activity are demonstrated with in the Mn1 and Mn7 clones. Proteins (250 μg each) were separated on native polyacrylamide gels and stained for MnSOD activity by the photoinduced NBT reaction with 0.75 mM sodium cyanide. All activity gels were performed in duplicate.

control cell lines had approximately equal amounts of immunoreactive MnSOD protein. However, the two MnSOD-transfected cell lines had 1.5- and 2.1-fold increases in immunoreactive protein compared to the MIA PaCa-2 cell line. Densitometric quantitation of individual MnSOD bands is shown at the bottom of the Western blot. Likewise, the activity gels demonstrated similar increases in MnSOD activity in the Mn1 and Mn7 clones when compared to the parental wild-type and neo control cell lines (Fig. 1B). There were no significant changes in immunoreactive CuZnSOD activity in the cell lines as measured by the activity gels.

Antioxidant Enzyme Activities

Because of the importance of both anitoxidant enzyme activity and the balance among antioxidant enzymes, we measured the four major mammalian antioxidant enzymes (MnSOD, CuZnSOD, catalase and GPx) in the pancreatic cancer cell lines. Enzyme activity is the most important parameter to determine the function of MnSOD because the expression of MnSOD mRNA may not necessarily mean an increase in MnSOD protein.[22] Moreover, even increased MnSOD protein is not necessarily active. Therefore, we performed both enzymatic gels and activity assays to measure the activity of MnSOD. Table I demonstrates that there were no significant differences in the enzyme activities of CuZnSOD, catalase or GPx in the wild-type, vector control or MnSOD-overexpressing cell lines. In contrast, all the MnSOD-transfected cell lines had a 2–3-fold increase in MnSOD activity. The MnSOD enzyme activity assays correlated well ($r^2 = 0.91$, $P < 0.05$) with the results obtained with Western blotting. It should be emphasized at this point that enzymatic activity data were calculated from multiple samples, while the western blots and activity gels discussed previously were from only one sample (different samples for the western blots and activity gels). Unfortunately, the neo clone selected also had slightly increased MnSOD activity. This could be due to several reasons: pre-existing cells with high MnSOD, stress-induction, etc. This is not a significant problem since in many of the following studies, we used correlation analysis to determine if MnSOD played a role.

Tumor Biological Characteristics of MnSOD-overexpressing Cells

Cell Growth

MnSOD-overexpressing cells demonstrated slower *in vitro* growth compared to parental cells (Fig. 2A). MIA PaCa-2 cell doubling time significantly increased with the Mn7 clone when compared to the parental cells or the vector control cells. Tumor cell doubling time was 26 h for the parental cell line and 29 h in the neo controls. In the MnSOD-overexpressing cell lines, doubling time increased to 40 and 53 h in the Mn1 and Mn7 clones, respectively (Fig. 2B). For example, at 96 h, cell number decreased approximately by 45% with the Mn1 cell line and by approximately 64% with Mn7 cell line when compared to the neo controls (Fig. 2A).

Plating Efficiency

To determine the clonogenic capacity of MnSOD overexpressing cells, we performed a plating efficiency assay. In general, malignant cells have a higher plating efficiency than do normal cells. Plating efficiency was reduced in the MnSOD transfected cells compared with the parental cells (Fig. 3A). Plating efficiency was 16.7 ± 1.9 and $17.3 \pm 0.4\%$ in the wild type and neo control cell lines, respectively. Transfection of MnSOD decreased the plating efficiency to $11.2 \pm 0.4\%$ in the Mn1 clones and $5.1 \pm 0.2\%$ in the Mn7 clones ($P < 0.01$ vs. neo controls).

Growth in Soft Agar

To examine anchorage-dependent growth, we performed a soft agar assay. Whereas malignant cells form colonies in soft agar, normal cells do so in far smaller numbers. Overexpression of MnSOD significantly reduced colony formation (Fig. 3B). Soft agar plating efficiency was 0.92 ± 0.15 and $0.75 \pm 0.12\%$ in the wild type and neo controls, respectively. Colony formation was significantly reduced with the Mn1 clones to 0.17 ± 0.15 and to 0.21 ± 0.11 in the Mn7 clones (Mean $\pm$ SEM, $P < 0.01$ vs. neo controls). There was an inverse correlation with the MnSOD enzyme activity assays and anchorage-dependent growth in soft agar ($r^2 = 0.91$, $P < 0.05$).

TABLE I Antioxidant enzyme activity in MIA PaCa-2 cell lines

Cell line	MnSOD (units/mg protein)	CuZnSOD (units/mg protein)	Catalase (mk units/mg protein)	GPx (munits/mg protein)
MIA PaCa-2	13.3 ± 2.0	46.5 ± 7.7	6.9 ± 2.9	15.5 ± 3.3
Neo control	22.0 ± 0.5	68.6 ± 5.9	6.3 ± 2.1	15.0 ± 3.3
Mn1	36.8 ± 7.2*	48.5 ± 12.0	4.9 ± 0.7	22.8 ± 1.3
Mn7	29.9 ± 3.2*	62.0 ± 10.1	3.3 ± 0.7	19.2 ± 1.6

Values are mean ± SEM, $n = 3$. *$P < 0.05$ compared to the neo control, MIA PaCa-2.

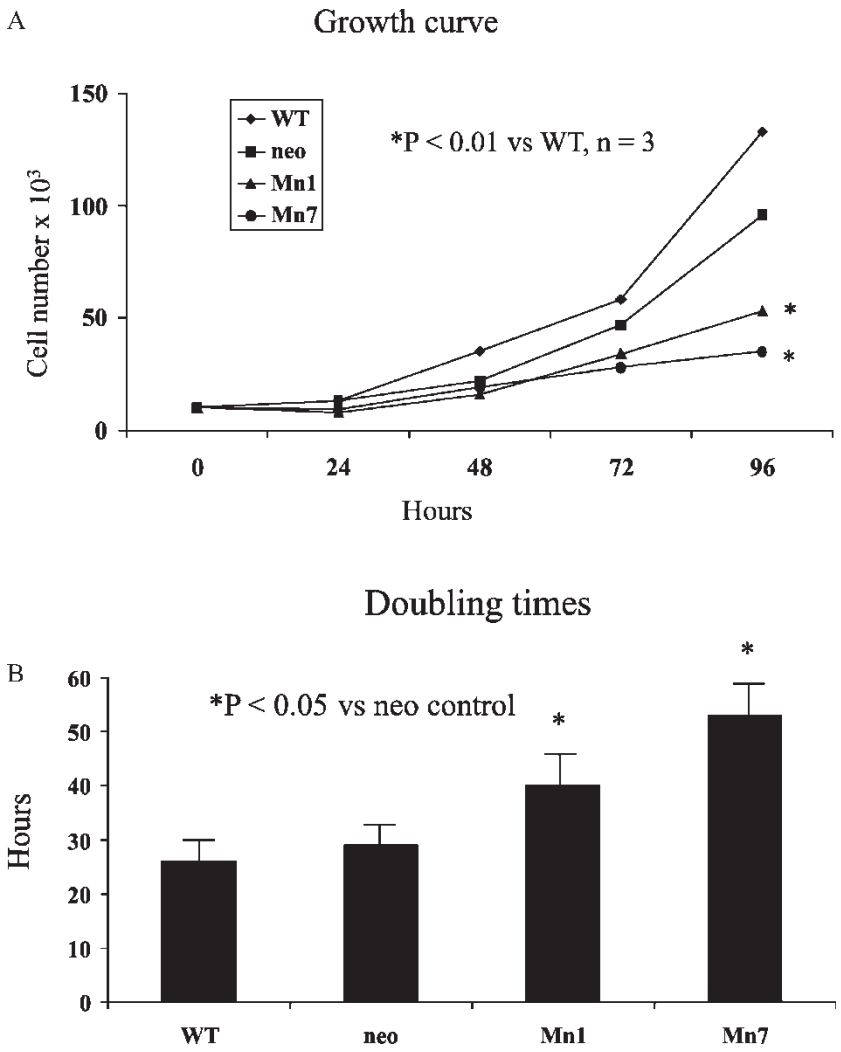

FIGURE 2 (A) Cell growth. MIA PaCa-2 cells stably transfected with MnSOD demonstrate reductions in cell growth. No significant changes were seen with the V2B (neo control) compared with parental cells (wild-type). Mean *in vitro* cell growth is shown. Each point was determined in triplicate. *$P < 0.01$ vs. WT. (B) Doubling times. MIA PaCa-2 cells stably transfected with MnSOD demonstrate increases in doubling times. No significant changes in doubling time were seen with the V2B (neo control) compared with parental cells (wild-type). Mean doubling time is shown. Each point was determined in triplicate. *$P < 0.01$ vs. WT.

Effect of MnSOD Overexpression on Sensitivity to Increased Intracellular Production of $O_2^{\cdot-}$.

Effect of MnSOD Overexpression on Radiation Response

Cell radiosensitivity was determined first by the MTT assay (Fig. 4A). In general, MnSOD-overexpressing cell lines were more radioresistant than the MIA PaCa-2 parental cell line. The neo control cell line also demonstrated radioresistance compared to the parental cell line at 2, 4 and 6 Gy. However, the Mn1 and Mn7 clones had significantly increased radioresistance at the 4 Gy dose when compared to the neo control cell line. Unlike the MTT assay, the clonogenic cell survival assay (Fig. 4B) demonstrated radiosensitivity in the MnSOD overexpressing clones. There was a significant decrease in colony formation in the Mn1 and Mn7 cell line at doses of 2 and 4 Gy. At 6 Gy there was no colony formation in any of the cell lines. Although, the observed differences in the MnSOD-overexpressing cell lines may be due to growth rate or cytostatic effects, the lack of colonies in these cell lines after radiation suggests a cytotoxic effect.

Effect of MnSOD Overexpression on Dicumarol Treatment

Recent studies have demonstrated that the two-electron reductase enzyme NADPH:quinone oxidoreductase is upregulated in pancreatic cancer[23] and pancreatic cancer cell lines.[10] Inhibition of this enzyme with the competitive, specific inhibitor dicumarol results in increased semiquinone production. Semiquinones react with molecular oxygen to form intracellular $O_2^{\cdot-}$.[10]. Cell viability with dicumarol treatment was first determined by the MTT assay (Fig. 5A). In a similar pattern as seen with

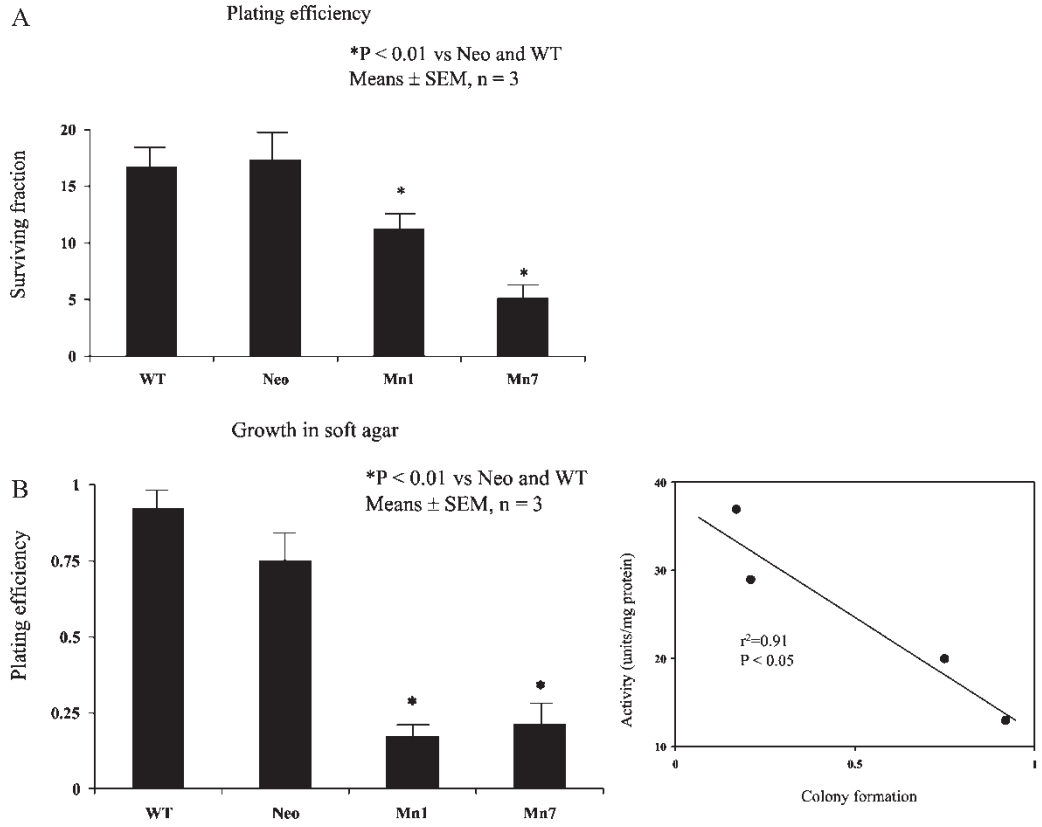

FIGURE 3 (A) Plating efficiency. MIA PaCa-2 cells stably transfected with MnSOD demonstrate reductions in plating efficiency. No significant changes were seen with neo controls compared with wild-type parental cells. Mean plating efficiency of wild-type, neo, Mn1 or Mn7 cells are shown. Each determination was performed in triplicate. $P < 0.05$ vs. wild-type. (B) Growth in soft agar. MIA PaCa-2 cells stably transfected with MnSOD demonstrate reductions in soft agar plating efficiency. No significant changes were seen with neo controls compared with wild-type parental cells. Mean plating efficiency in soft agar of wild-type, neo controls, Mn1 or Mn7 cells are shown. Each determination was performed in triplicate. $*P < 0.01$ vs. wild-type or neo. Linear regression analysis indicated an inverse correlation with the MnSOD enzyme activity assays and anchorage-dependent growth in soft agar.

γ radiation, dicumarol treatment resulted in increased cell viability in the MnSOD overexpressing clones as measured by MTT, but decreased clonogenic survival, when compared to the parental cell lines. Dicumarol, 100 μM for 4 h resulted in increased cell viability in the Mn1 clone while dicumarol 100 μM for 24 h resulted in increased cell viability in both the Mn1 and Mn7 clones when compared to the parental cell lines. Unlike the MTT assay, the clonogenic cell survival assay (Fig. 5B) demonstrated decreased clonogenic survival in the Mn7 MnSOD overexpressing clone. At a dicumarol dose of 100 μM for 24 h, there were no surviving colonies ($P < 0.05$ vs. neo control cell line).

Tumorigenicity in Nude Mice

Tumor volume was compared among the four groups using the data up to day 28 only. To compare treatment groups over time for tumor volume, the linear mixed model analysis[20] assuming a compound symmetry covariance structure for within subjects was used. In the linear mixed model analysis, group was considered a fixed effect and day was considered a continuous covariate. An interaction term between day and group was also included in the model. Using day as a continuous covariate assumes that the mean tumor volume is a linear function of days. The linear assumption was acceptable since the adjusted r^2s were all at least 0.79 (wild type: $r^2 = 0.92$; neo: $r^2 = 0.79$; Mn1: $r^2 = 0.88$; Mn7 $r^2 = 0.90$). The linear mixed model suggested that the interaction between day and group was statistically significant ($P = 0.017$); that is, there was an overall difference among the groups in the slopes for tumor volume over time. The estimates for the slopes by group are provided in Table II.[21] As indicated in Fig. 6, there were significant differences between wild-type and the Mn1 and Mn7 clones. Additionally, there were significant differences between the neo controls and the Mn1 group of animals. Thus, MnSOD overexpression caused the pancreatic cancer cell line to grow much slower *in vivo*. There was an inverse correlation between the MnSOD enzyme activity assays ($r^2 = 0.96$, $P < 0.05$) and the tumor size at day 28, i.e. the higher the MnSOD activity, the slower *in vivo* tumor growth.

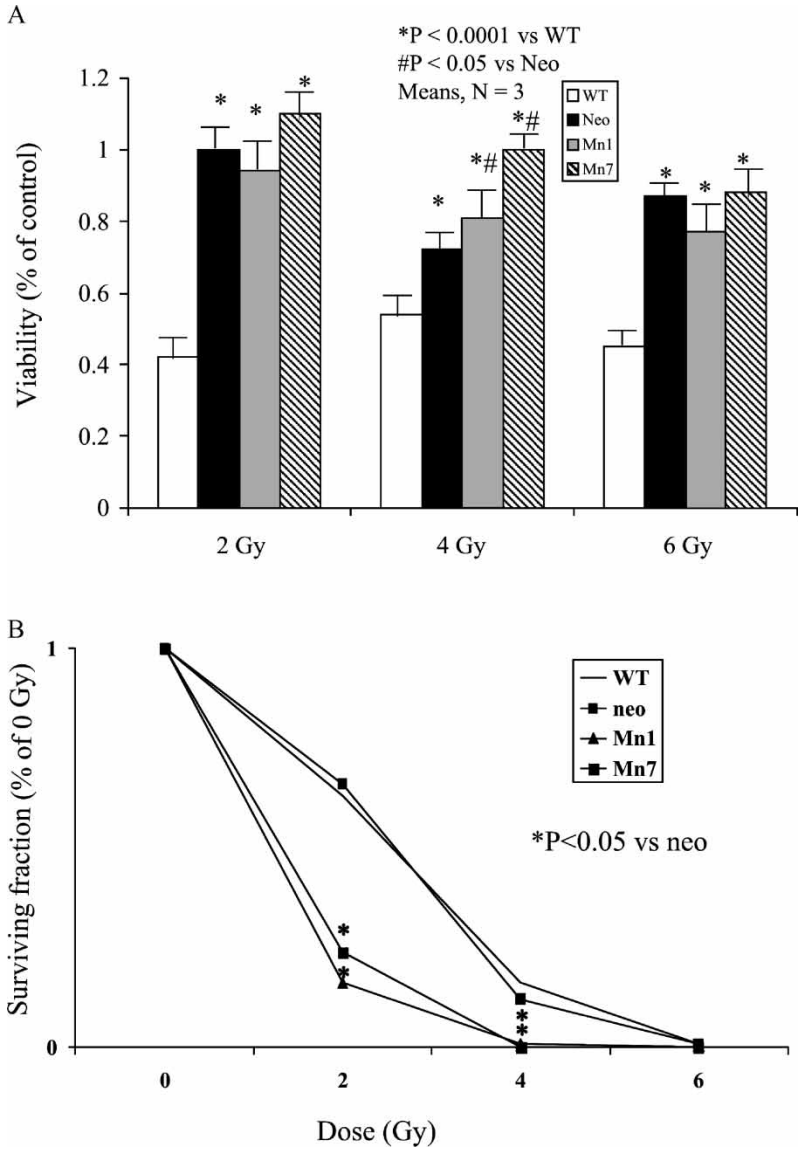

FIGURE 4 Effect of MnSOD overexpression on radiosensitivity of MIA PaCa-2 cells. (A) MTT assay of radiation survival. Cells were seeded at 3000 cells/well in 96-well plates overnight and exposed to 0, 2, 4 or 6 Gy of radiation from a ^{137}Cs source. After irradiation, metabolic cell viability was determined by the MTT assay on day 3. Means, $N = 3$, $*P < 0.0001$ vs. WT, $P < 0.05$ vs. Neo. (B) Clonogenic assay of radiation survival. Cells were seeded at 300 cells/60-mm culture dish were exposed to 0, 2, 4 or 6 Gy of radiation from a ^{137}Cs source. Surviving fraction was determined at 1 week. Means, $N = 3$, $*P < 0.05$ vs. neo.

DISCUSSION

Our study demonstrated that stable overexpression of MnSOD decreased *in vitro* growth rates, growth in soft agar and plating efficiency, while *in vivo*, MnSOD-overexpressing clones had slower growth in nude mice. Combining MnSOD overexpression with other modalities that increase production of $O_2^{\cdot-}$ (i.e. radiation and dicumarol) increased cytotoxicity as measured by the clonogenic cell survival assay. Taken together with other studies from our laboratory demonstrating suppression of the malignant phentoype after infecting pancreatic cancer cells with an adenovirus-MnSOD construct,[4] the present studies with stable overexpression provide further evidence that MnSOD may be a tumor suppressor gene in human pancreatic cancer.

Our results correlate with other studies of MnSOD overexpression in other cell lines. In rat glioma cells, MnSOD overexpression inhibited growth compared to parental and vector control cells.[9] Additionally, MnSOD overexpression sensitized cells to radiation and inhibitors of hydroperoxide removal. In our present study, the clonogenic cell survival assay demonstrated that MnSOD overexpression also rendered cells more sensitive to increased $O_2^{\cdot-}$ levels presumably by increasing intracellular H_2O_2 production which in turn is converted to hydroxyl radicals to cause cell killing.

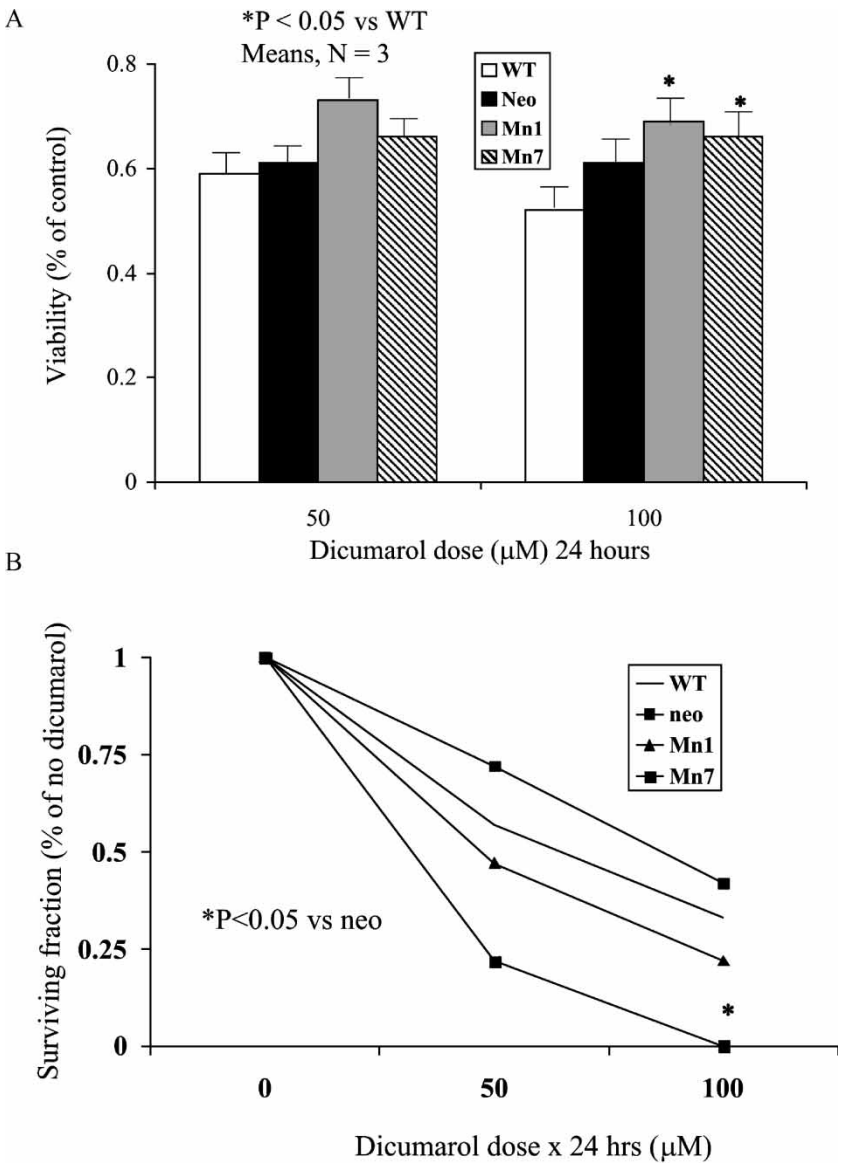

FIGURE 5 Effect of MnSOD overexpression on dicumarol response in MIA PaCa-2 cells. (A) MTT assay of metabolic viability after dicumarol treatment. Cells were seeded at 3000 cells/well in 96-well plates overnight and treated with dicumarol 0, 50 or 100 μM for 24 h. Metabolic cell viability was determined by the MTT assay on day 2. Means, $N = 3$, $*P < 0.05$ vs. wild-type. (B) Clonogenic assay after dicumarol treatment. Cells were seeded and exposed to dicumarol 0, 50 or 100 μM for 24 h. Surviving fraction was determined at 1 week. Means, $N = 3$, $*P < 0.05$ vs. neo.

In contrast to MnSOD overexpression in glioma cells, our study demonstrates that MnSOD overexpression in pancreatic cancer cells increases metabolic cell viability as measured by MTT. This is consistent with the premise that MnSOD is decreasing levels of $O_2^{\cdot-}$ by dismutation to H_2O_2.

TABLE II *In vivo* tumor volume slopes over time for the data shown in Figure 5

Group	Slope (CI 95%)
Wild type	17.3 (11.5–23.0)
Neo	13.4 (7.6–19.1)
Mn1	4.8 (−1.0–10.6)
Mn7	7.4 (1.–13.1)

The MTT assay widely used in cell proliferation and cytotoxicity assays, uses electron acceptors to facilitate reduction of formazans.[24] Our study demonstrated that MnSOD overexpression inhibited reduction of formazans indicating involvement of $O_2^{\cdot-}$ in the reductive mechanism. MnSOD decreases levels of $O_2^{\cdot-}$ by conversion to H_2O_2. H_2O_2 is well known for its cytotoxic effect leading to cell death as seen in the clonogenic assay. Collier and Pritsos have recently demonstrated similar results with the different effects of dicumarol when measured by the MTT assay and the clonogenic assay.[25] In mouse mammary tumors and Chinese hamster ovary cells, significant increases in cell viability were demonstrated after short time period treatments with

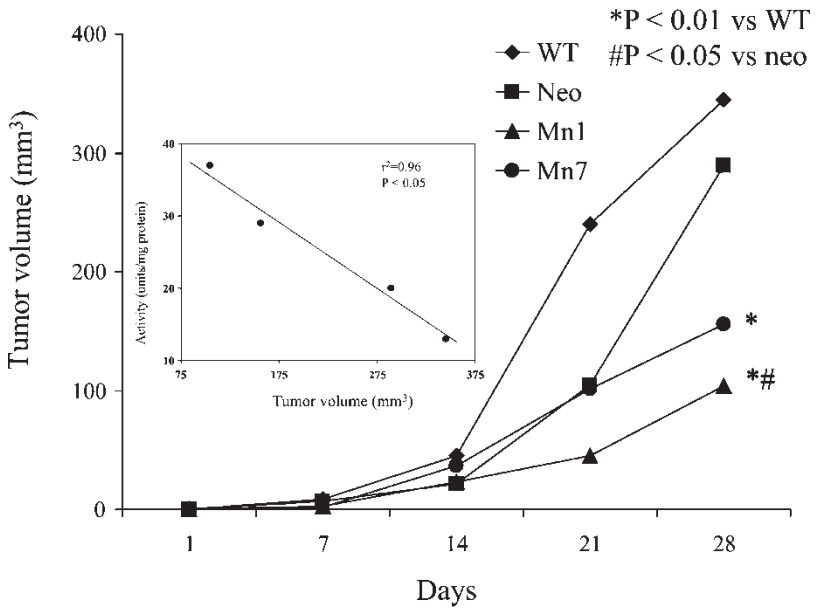

FIGURE 6 MnSOD overexpression decreased MIA PaCa-2 tumor growth in nude mice. Cells (2×10^6) were injected subcutaneously in the flank region of nude mice and tumor size was measured once a week. MIA PaCa-2 overexpressing MnSOD (Mn1 and Mn7) tumors showed a 2–3-fold decrease in tumor growth over the wild-type tumors or neo controls (Day 28: median tumor volume 345 mm^3 in wild-type tumors and 289 mm^3 in neo controls vs. 104 mm^3 in Mn1 and 156 mm^3 in Mn7). $*P < 0.01$ vs. WT, $P < 0.05$ vs. neo. Linear regression analysis indicated an inverse correlation with the MnSOD enzyme activity assays and tumor volume on day 28.

dicumarol. Clonogenic assays provided contrasting results where greater dicumarol toxicity was observed when compared to the MTT assay. Additionally, ROS production in these cell lines was increased in a concentration-dependent manner with dicumarol administration. The MTT assay in our current study also demonstrated that the vector control cells are resistant to radiation but not to dicumarol. The generation of ROS after dicumarol treatment is currently under investigation in our laboratory. The molecule or molecules involved in the ROS effects following dicumarol treatment are unknown. There are two obvious possibilities whereupon dicumarol treatment could induce ROS generation. First, NAD(P)H:quinone oxidoreductase (NQO_1) directly reduces quinones to the hydroquinone form, thus bypassing the reactive semiquinone intermediate during metabolism.[26–29] Dicumarol inhibits NQO_1, initiating ROS production when reactive semiquinones generate a redox cycle, resulting in superoxide ($O_2^{\cdot-}$) formation. Dicumarol also inhibits the quinone form of coenzyme Q resulting in semiquinone intermediates that combine with molecular O_2 to form $O_2^{\cdot-}$.[25,30] The net result is an increase in ROS following dicumarol treatment.

Although our study demonstrates that MnSOD overexpression inhibits pancreatic cell growth, other investigations have demonstrated that MnSOD has antiapoptotic effects in cellular protection. Park *et al.* demonstrated that mitochondrial DNA-depleted SK-Hep1 rho (0) hepatoma cells were resistant to apoptosis and MnSOD expression was profoundly increased in these rho (0) cells.[31] Also, previous studies in a human ovarian cancer cell line demonstrated that overexpression of MnSOD inhibited cell growth but induced irradiation resistance than parental cells.[32]

The results from this study demonstrate that overexpression of MnSOD can enhance the antitumor effects of radiation and dicumarol. These treatment combinations could be successful *in vivo*. The proposed mechanism for these effects is the increased H_2O_2 production from MnSOD dismutation, which cannot be detoxified by the levels of catalase or GPx. H_2O_2 is then free to diffuse to nearby cells and, through Fenton chemistry, is converted to hydroxyl radical to cause cellular toxicity and death. The killing observed in the clonogenic cell survival is postulated to rely on the fact that the H_2O_2-removing antioxidant enzymes catalase and GPx, are not also increased. The mechanism by which H_2O_2 might inhibit tumor growth is still unknown. However, Weydert *et al.* have demonstrated cell killing via both apoptotic and necrotic mechanisms *in vitro*, but the *in vivo* mechanism is unclear.[8]

In summary, overexpression of MnSOD suppresses the malignant phenotype of pancreatic cancer, while MnSOD-overexpressing clones had slower growth in nude mice. Combining MnSOD overexpression with other modalities that increase intracellular production of $O_2^{\cdot-}$ increased cytotoxicity presumably by increasing levels of H_2O_2. These results provide further evidence that MnSOD may be a tumor suppressor gene in human pancreatic cancer.

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References

[1] McCord, J.M., Keele, B.B. and Fridovich, I. (1971) "An enzyme based theory of obligate anaerobiosis: the physiological function of superoxide dismutase", *Proc. Natl Acad. Sci. USA* **68**, 1024–1027.
[2] Cullen, J.J., Mitros, F.A. and Oberley, L.W. (2003) "Expression of antioxidant enzymes in diseases of the human pancreas: another link between chronic pancreatitis and pancreatic cancer", *Pancreas* **26**, 23–27.
[3] Cullen, J.J., Weydert, C., Hinkhouse, M.M., Ritchie, J.M., Domann, F.E., Spitz, D.R. and Oberley, L.W. (2003) "The role of manganese superoxide dismutase in the growth of pancreatic adenocarcinoma", *Cancer Res.* **63**, 1297–1303.
[4] Wydert, C., Roling, B., Liu, J., Ritchie, J.M., Oberley, L.W. and Cullen, J.J. (2003) "Suppression of the malignant phenotype in human pancreatic cancer cells by the overexpression of manganese superoxide dismutase", *Mol. Cancer Ther.* **2**, 361–369.
[5] Jemal, A., Thomas, A., Murray, T. and Thun, M. (2002) "Cancer statistics, 2002", *CA Cancer J. Clin.* **52**, 23–47.
[6] Yeo, C.J. and Cameron, J.L. (1999) "Pancreatic cancer", *Curr. Probl. Surg.* **36**, 59–152.
[7] Li, S., Yan, T., Yang, J.Q., Oberley, T.D. and Oberley, L.W. (2001) "The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase", *Cancer Res.* **60**, 3927–3939.
[8] Weydert, C.J.D., Smith, B., Xu, L., Kregel, K.C., Ritchie, J.M., Davis, C.S. and Oberley, L.W. (2003) "Inhibition of oral cancer cell growth by adenovirus MnSOD plus BCNU treatment", *Free Radic. Biol. Med.* **34**, 316–329.
[9] Zhong, W., Oberley, L.W., Oberley, T.D., Yan, T., Domann, F.E. and St. Clair, D.K. (1996) "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.
[10] Cullen, J.J., Hinkhouse, M.M., Grady, M., Gaut, A.W., Liu, J., Zhang, Y., Weydert, C.J.D., Domann, F.E. and Oberley, L.W. (2003) "Dicumarol inhibition of NAD(P)H:quinone oxidoreductase (NQO_1) induces growth inhibition of pancreatic cancer via a superoxide-mediated mechanism", *Cancer Res.* **63**, 5513–5520.
[11] Li, J.J., Oberley, L.W., St. Clair, D.K., Ridnour, L.A. and Oberley, T.D. (1995) "Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase", *Oncogene* **10**, 1989–2000.
[12] Oberley, T.D., Oberley, L.W., Slattery, A.F., Lauchner, L.J. and Elwell, J.H. (1990) "Immunohistochemical localization of antioxidant enzymes in adult Syrian hamster tissues and during kidney development", *Am. J. Pathol.* **137**, 199–214.
[13] Spitz, D.R. and Oberley, L.W. (1989) "An assay for superoxide dismutase in mammalian tissue homogenates", *Anal. Biochem.* **179**, 8–18.
[14] Lawrence, R.A. and Burk, R.F. (1976) "Glutathione peroxidase activity in selenium-deficient liver", *Biochem. Biophys. Res. Commun.* **71**, 952–958.
[15] Beers, R.F., Jr. and Sizer, I.W. (1952) "A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase", *J. Biol. Chem.* **195**, 133–140.
[16] Aebi, H. (1984) "Catalase *in vitro*", In: Packer, L., ed., *Methods in Enzymology* (Academic Press, New York), Vol. 105, pp 121–126.
[17] Davis, B.J. (1964) "Disc electrophoresis-II. Method and application to human serum proteins", *Ann. NY Acad. Sci.* **121**, 404–427.
[18] Beauchamp, C. and Fridovich, I. (1971) "Superoxide dismutase: improved assays and an assay applicable to acrylamide gels", *Anal. Biochem.* **44**, 276–287.
[19] Lowry, O.H., Rosebrough, N.G. and Randall, R.J. (1951) "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.
[20] Littell, R.C., Milliken, G.A., Stroup, W.W. and Wolfinger, R.D. (1996) *SAS System for Mixed Model* (SAS Institute Inc., Cary, NC).
[21] Akaike, H. (1973) "Information theory and an extension of the maximum likelihood principle", In: Petrov, B.N. and Csaki, F., eds, *Second International Symposium on Information Theory* (Akademiai Kiado, Budapest), pp 267–281.
[22] Czaja, M.J., Schilsky, M.L., Xu, Y., Schmiedeberg, P., Compton, A., Ridnour, L. and Oberley, L.W. (1994) "Induction of MnSOD gene expression in a hepatic model of TNF-α toxicity does not result in increased protein", *Am. J. Physiol.* **266**, G737–G744.
[23] Logsdon, C.D., Simeone, D.M., Binkley, C., Arumugam, T., Greenson, J.K., Giordano, T.J., Misek, D.E. and Hanash, S. (2003) "Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer", *Cancer Res.* **63**, 2649–2657.
[24] Berridge, M.V., Tan, A.S., McCoy, K.D. and Wang, R. (1996) "The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts", *Biochemica* **4**, 14–19.
[25] Collier, A.C. and Pritsos, C.A. (2003) "The mitochondrial uncoupler dicumarol disrupts the MTT assay", *Biochem. Pharmacol.* **66**, 281–287.
[26] Trush, M.A., Twerdok, L.E., Rembish, S.J., Zhu, H. and Li, Y.B. (1996) "Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities", *Envrion. Health Perspect.* **104**(Suppl. 6), 1227–1234.
[27] Pink, J.J., Planchon, S.M., Tagliarino, C., Varnes, M.E., Siegel, D. and Boothman, D.A. (2000) "NAD(P)H:Quinone oxidoreductase activity is the principal determinant of β-lapachone cytotoxicity", *J. Biol. Chem.* **275**, 5416–5424.
[28] Siegel, D. and Ross, D. (2000) "Immunodetection of NQO1 in human tissues", *Free Radic. Biol. Med.* **29**, 246–253.
[29] Llopis, J., Ernster, L. and Cadenas, E. (1990) "Effect of glutathione on the redox transitions of naphthohydroquinone derivatives fromed during DT-diaphorase catalysis", *Free Radic. Res. Commun.* **8**, 271–285.
[30] Santos-Ocana, C., Villalba, J.M., Cordoba, F., Padilla, S., Crane, F.L., Clarke, C.F. and Navas, P. (1998) "Genetic evidence for coenzyme Q requirement in plasma membrane electron transport", *J. Bioenerg. Biomembr.* **5**, 465–475.
[31] Park, S.Y., Chang, I., Kim, J.Y., Kang, S.W., Park, S.H., Singh, K. and Lee, M.S. (2004) "Resistance of mitochondrial DNA-depleted cells against cell death. Role of mitochondrial superoxide dismutase", *J. Biol. Chem.* **279**, 7512–7520.
[32] Takada, Y., Hachiya, M., Park, S.H., Osawa, Y., Ozawa, T. and Akashi, M. (2002) "Role of reactive oxygen species in cells overexpressing manganese superoxide dismutase: mechanism for induction of radioresistance", *Mol. Cancer Res.* **2**, 137–146.