RESEARCH ARTICLE

Bidirectional Regulation of Manganese Superoxide Dismutase (MnSOD) on the Radiosensitivity of Esophageal Cancer Cells

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Abstract

The mitochondrial antioxidant protein manganese superoxide dismutase (MnSOD) may represent a new type of tumor suppressor protein. Overexpression of the cDNA of this gene by plasmid or recombinant lentiviral transfection in various types of cancer leads to growth suppression both in vitro and in vivo. We previously determined that changes in MnSOD expression had bidirectional effects on adriamycin (ADR) when combined with nitric oxide (NO). Radiation induces free radicals in a manner similar to ADR, so we speculated that MnSOD combined with NO would also have a bidirectional effect on cellular radiosensitivity. To examine this hypothesis, TE-1 human esophageal squamous carcinoma cells were stably transfected using lipofectamine with a pLenti6–DEST plasmid containing human MnSOD cDNA at moderate to high overexpression levels or with no MnSOD insert. Blastidicin-resistant colonies were isolated, grown, and maintained in culture. We found that moderate overexpression of MnSOD decreased growth rates, plating efficiency, and increased apoptosis. However, high overexpression increased growth rates, plating efficiency, and decreased apoptosis. When combined with NO, moderate overexpression of MnSOD increased the radiosensitivity of esophageal cancer cells, whereas high MnSOD overexpression had the opposite effect. This finding suggests a potential new method to kill certain radioresistant tumors and to provide radioresistance to normal cells.

Keywords: Manganese superoxide dismutase - nitric oxide - esophageal carcinoma - reactive oxygen species

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Introduction

Manganese superoxide dismutase (MnSOD) is a newly recognized anti-oncogene with low expression or detection in breast cancer (Soini et al., 2001), prostatic cancer (Wang et al., 2006), lung cancer (Zejnilovic et al., 2009), and esophageal cancer (Sun et al., 2011). Interestingly, the overexpression of MnSOD reduced metastasis rates (Wang et al., 2005) and inhibited or reversed the malignant phenotype of human fibroblast SV40 (Yan et al., 1996), malignant melanoma (Church et al., 1993)and neuroglioma (Zhong et al., 1997). However, we found that overexpression of MnSOD did not always inhibit tumor growth. High MnSOD overexpression inhibited the growth of SaOS2 osteosarcoma cells, producing low inoculation efficiency with a longer doubling time, whereas moderate overexpression produced high inoculation efficiency and a shorter doubling time (Wang et al., 2005). We speculated that by regulating the levels of MnSOD overexpression, in the presence of nitric oxide (NO) and adriamycin (ADR), would alter the types and concentrations of reactive oxygen species (ROS) and reaction products in a biodirectional manner (Wang et al., 2005). We previously showed that protein expression levels of MnSOD in esophageal carcinoma cells were lower than those of normal tissues (Sun et al., 2011), and established TE-1 esophageal cancer cell lines to express MnSOD at different concentrations. Using radiation and NO, we evaluated whether the expression levels of MnSOD may affect the biological behavior of esophageal cancer cells, and also elucidate the mechanism of action using an ROS assay.

Materials and Methods

Main reagents

All reagents were commercially available and purchased as follows: Sodium nitroprusside (SNP), bovine serum albumin (BSA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies were purchased from Sigma Chemical Company (St. Louis, MO, USA). Plenti6/V5–DEST Vector, Lipofectamine 2000, and the SuperScript III Reverse Transcriptase (RT) kit were obtained from Invitrogen Corporation (Carlsbad, CA, USA). A rabbit anti-human MnSOD monoclonal antibody was bought from Epitomics, Inc. (Burlingame, CA, USA). TaqDNA polymerase were purchased from Fermentas, Inc. (Waltham, MA, USA). DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR Bioscience, Inc. (Lincoln, Nebraska, USA).

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An immunohistochemistry kit and an Annexin V-FITC/PI apoptosis detection kit were purchased from 4A Biotech Co. Ltd. (Beijing, China). An ROS assay kit and 3-amino,4-aminomethyl-2',7'-difluorescein diacetate were purchased from Beyotime Co. (Jingsu, China). Fetal bovine serum (FBS), cell-culture media, and supplementary materials were obtained from Gibco Co. (Grand Island, NY, USA).

Cell culture and transfection

TE-1 cells, from a human esophageal squamous carcinoma cell line, were obtained from Dr. BE Shan (Department of Scientific Research Center, Fourth Hospital of Hebei Medical University, Shijiazhuang, China,). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, and were negative for mycoplasma in a humidified 5% carbon dioxide (CO_2) atmosphere at 37 ºC. Medium was changed every 2-3 days. When the cultures reached confluence, the cells were sub-cultured with 0.25% trypsin and 1% ethylenediaminetetraacetic acid. Each sub-culture was transfected with a moderate or high level of MnSOD. The selection marker control was transfected with the plenti6/V5-DEST vector. After 24 h of cultivation, culture medium was changed and the antibiotic Blastidicin (5 μg/ml) was added. Ten days later, three cells were selected, cloned, and inoculated into a 24-pore plate. After full growth of the cells, cultures were enlarged to select cell lines with stable transfection.

Immunofluorescent staining

Immunofluorescence was measured as previously described (Weydert et al., 2006; Zhang et al., 2006; Ressel et al., 2010). About 1×10^6 cells were collected and washed twice with ice-cold PBS. The harvested cells were transferred to slides, fixed for 30 min in freshly prepared 4% formaldehyde, and subjected to permeabilization with 1% Triton X-100 for 10 min. The cells were incubated with a rabbit anti-human MnSOD monoclonal antibody (1:100 dilution) at 4 ºC overnight and a CY3 conjugated goat anti-rabbit IgG (1:100 dilution) for 1 h at room temperature. Image observation and capture were immediately performed using a confocal microscope (Nikon, Tokyo, Japan).

Reverse transcription polymerase chain reaction (RT–PCR) analysis

After the specimen was removed from liquid nitrogen, total RNA was extracted according to the instructions on Trizol reagent (Invitrogen). A total of 2 μg RNA was used to synthesize cDNA in a 20 μl reaction system, according to the instructions of the Superscript III Reverse Transcriptase kit. The reaction conditions were as follows: denaturation at 65ºC for 5 min and RT at 50 ºC for 50 min. PCR amplification was performed on the MnSOD gene upstream primer (5'-AAGGTCGGAGTCAACGGATT-3') and downstream primer (5'-GCTCCTGGAAGAT GGTGAT-3'), producing an amplified fragment of 158 bp. Additionally, amplification of the GAPDH gene upstream primer (5'-CCGACCTGCCCTACGACTA-3') and downstream primer (5'-CTGGGCTGTAACATCTCCCTT-3') produced an amplified fragment with a length of 226 bp. The PCR reaction included 5 μl 10X PCR buffer, 1 μl 10 mmol dNTP, 0.5 μl TaqDNA polymerase, 2 μl each of the upstream and downstream primers, 2 μl template cDNA, and ddH_2O to reach a total volume of 50 μl. Reaction conditions were as follows: pre-denaturation at 95 ºC for 3 min, denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s, and extension at 72 ºC for 45 s for a total of 35 cycles at -20 ºC. After gel electrophoresis in 1.5% agarose, ethidium bromide-stained bands were visualized by ultraviolet transillumination and the fluorescence intensity was quantified using a Gel Doc 2000 system (BioRad, CA, USA). Under these experimental conditions, the incorporated fluorescence intensity showed that the PCR products for MnSOD and GAPDH were all within the linear phase of the reaction using 30 PCR cycles.

Western blot analysis

To determine MnSOD immunoreactive protein levels, cells were grown to 70–75% confluence in 100-mm tissue culture plates. Cells were harvested by aspirating off the medium and washing twice in PBS, followed by suspension in 1 mL of PBS. After centrifugation, isolated cell pellets were lysed and sonicated in Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein was quantified using the Bradford protocol (Bradford, 1976). Denatured protein (50 μg) was then separated on 10% acrylamide gels by sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% nonfat dry milk in Tris-Buffered Saline and Tween-20 (TBST) and incubated with primary antibody (MnSOD, 1:500 dilution; GAPDH, 1:500 dilution) in 5% nonfat dry milk for 1 h at room temperature. After washing, the membrane was incubated with a goat anti-rabbit fluorescent secondary antibody (IRDye700, 1:20,000 dilution) in the dark for 1 h at room temperature. Blots were then scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, Nebraska, USA). Quantification was done by normalizing the signal intensity of each sample to that of GAPDH.

MTT cell proliferation assay

Cell viability was determined using the MTT (tetrazolium salt 3-4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/ml with 200 μl culture medium per well. After 24–72 h of culture, 20 μl of assay medium containing 5 mg/ml MTT was added to each well and incubated at 37 ºC for 4 h. The medium was gently aspirated, and then 150 μl of DMSO was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad, CA, USA) at 570 nm. The relative survival fraction of a tumor cell = (D value of the other three cells/ D value of TE-1 cell) × 100%.

Plating efficiency (PE)

PE was measured as previously described (Zhang et al., 2006). About 5×10^3 cells 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. PE was calculated as follows: PE = (colonies formed/number of cells seeded) × 100%.

Fluorescence-activated cell sorting (FACS) cell apoptosis analysis

FACS was performed according to manufacturer's instructions of the Annexin V-FITC apoptosis detection kit. Cells were collected, washed with ice-cold PBS, and re-suspended in binding buffer at a cell density of 1×10^6/mL. Cells were stained with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) (20 μg/mL) according to the manufacturer's instructions, then incubated in the dark at 25 ºC for 15 min. Samples were acquired on a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA), and 10,000 cells were analyzed with Cellquest software (Becton–Dickinson).

Radiosensitivity analysis

Radiosensitivity was determined according to the method described by Matthew and Antonella (Ough et al., 2004; Borrelli et al., 2009). Cells were plated in 96-well plates at a density that would allow cells to reach 70–75% confluency within 24 h. These cells were then separately treated with SNP (0–4 mmol/L), radiation (0–6 Gy of X-rays, linear accelerator, Elekta, Sweden), or 1 mmol/L SNP combined with 2 Gy radiation and allowed to grow for 48 h. After 48 h of culture, 20 μl assay medium containing 5 mg/ml MTT was added to each well and incubated at 37 ºC for 4 h. The medium was gently aspirated, and then 150 μl DMSO was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader at 570 nm. Inhibitory rate = 1 - (D value of experimental group/D value of control group) × 100%. Survival fraction (SF) = (D value of experimental group/D value of control group) × 100%. Multi-target single-hit of model-fitting cell curves was adopted, SF = $1 - (1 - e^{-D/D0})N$. Sensitization enhancement ratio (SER) = single radiation group D_0/sensitization group D_0.

Detection of intracellular ROS

ROS were measured with the non-fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Li et al., 2010). DCFH-DA passively diffuses into cells and is hydrolyzed by cellular esterases to 2',7'-DCFH, a non-fluorescent molecule that can be oxidized to the fluorescent molecule 2',7'- DCF in the presence of peroxides. Cells were seeded into 60-mm dishes and subjected to various treatments. Following treatment, cells were incubated with 10 mM DCFH-DA dissolved in cell-free medium at 37 ºC for 30 min, and then washed three times with PBS. DCF mean fluorescence intensity (MFI) was detected using a FACScan flow cytometer (Becton–Dickinson) and analyzed with Cellquest software (Becton–Dickinson). For each sample, 10,000 events were collected.

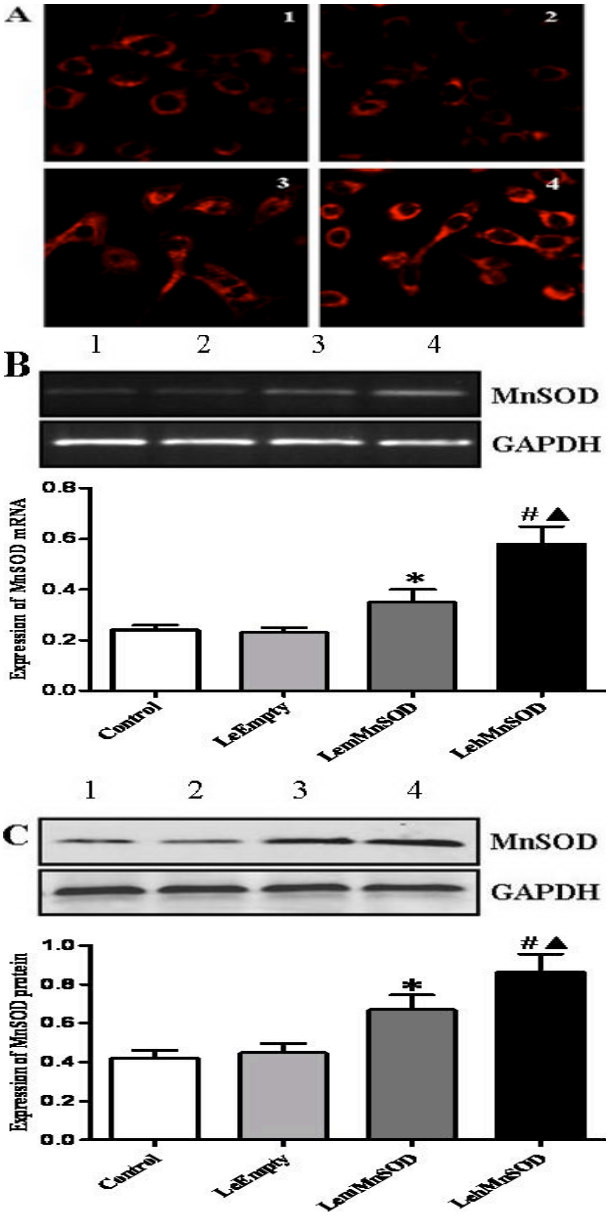

Figure 1. Expression and Identification of the Manganese Superoxide Dismutase (MnSOD) Gene. (A) Immunofluorescence images of TE-1 cells expressing MnSOD. Red fluorescent MnSOD protein stain in the cytoplasm of TE-1 cells observed with a fluorescence filter. (B) Reverse transcriptase polymerase chain reaction analysis for the medium to high overexpression of MnSOD mRNA compared with that of two untransfected cell lines. The expression of MnSOD mRNA was observed in a ~158-bp band. The relative abundances of MnSOD mRNA were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. *p = 0.000 compared to the two untransfected cells, #p = 0.002 compared to the two untransfected cell lines, ▲p = 0.039 compared to M-LeMnSOD transfected cells. (C) The Western blot shows the medium to high expression levels of cells with stable overexpression of MnSOD as compared to two untransfected cell lines. Total protein was electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel and a 5% stacking gel. The expression of MnSOD protein was observed in the ~26-kDa band. Western blot data configured as histograms are presented as means ± standard deviation of n = 3 in each group. *p = 0.011 compared to control cells and LeEmpty cells. #p = 0.000 compared to two untransfected cell lines. ▲p = 0.010 compared to M-LeMnSOD cells. 1, 2, 3, and 4 represent control, LeEmpty, M-LeMnSOD, and H-LeMnSOD cells, respectively

Statistical analysis

Data were expressed as means ± standard deviation (SD) unless otherwise specified. Student's unpaired t-tests were used to determine differences between two means. One-way analysis of variance (ANOVA) analysis with Tukey's post-analysis was used to assess the differences among three means. Three independent experiments were performed unless otherwise stated. Significance was determined at $p < 0.05$ and a 95% confidence interval. Statistical analysis was performed using SPSS13.0 for Windows (SPSS Inc., Chicago, IL, USA.). Partial graphs were created using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Stable transfection of MnSOD cDNA into TE-1 cells We performed stable transfection to introduce the pLenti6–MnSOD plasmid into TE-1 cells or the plenti6/V5-DEST vector alone as a control. After transfection, cells with high MnSOD overexpression (H-LeMnSOD cells), moderate MnSOD overexpression (M-LeMnSOD cells), and one neo-vector control cell (LeEmpty cell) were analyzed by immunofluorescence, as shown in Figure 1A. The control cells (TE-1 cells) and LeEmpty cells demonstrated little MnSOD protein fluorescence. M-LeMnSOD and H-LeMnSOD cells showed stronger protein fluorescence, suggesting that stably transfected cell lines with two levels of MnSOD overexpression were successfully constructed. To further examine transfection of MnSOD mRNA overexpression, cells were measured by RT–PCR, as shown in Figure 1B. MnSOD mRNA expression levels were 0.245 ± 0.024 and 0.236 ± 0.023 in two untransfected MnSOD cells, respectively. In contrast, M-LeMnSOD and H-LeMnSOD cells showed a large increase in mRNA expression, 0.354 ± 0.0510 and 0.587 ± 0.073, respectively. Western blot was also used to measure the amount of immunoreactive MnSOD protein in four cell lines (Figure 1C). The two untransfected cell lines had approximately equal amounts of immunoreactive protein, 0.359 ± 0.028 and 0.341 ± 0.022, respectively. However, the amount of immunoreactive protein in the MnSOD-transfected cell lines was 0.656 ± 0.048 and 0.838 ± 0.069, respectively.

Effects of MnSOD overexpression on cell proliferation

Cell viability was determined using the MTT assay. In general, the relative survival fraction of LeEmpty cells was approximately equal to that of control cells. However, M-LeMnSOD cells demonstrated slower in vitro growth compared to the two untransfected MnSOD cell lines at 24, 48, and 72 h. In contrast, there was a large increase in the relative survival fraction of H-LeMnSOD transfected cells compared to the two untransfected MnSOD cell lines. As for the MTT assay, there was almost no difference in the PE of the two untransfected MnSOD cell lines (34.7 ± 4.2 and 33.7 ± 4.8%, respectively). PE was reduced in the M-LeMnSOD cell line, which decreased the PE to 23.0 ± 2.6%. The H-LeMnSOD cells had a higher PE than normal cells, which increased the PE to 45.3 ± 4.5%. The apparent apoptosis of M-LeMnSOD cells was induced

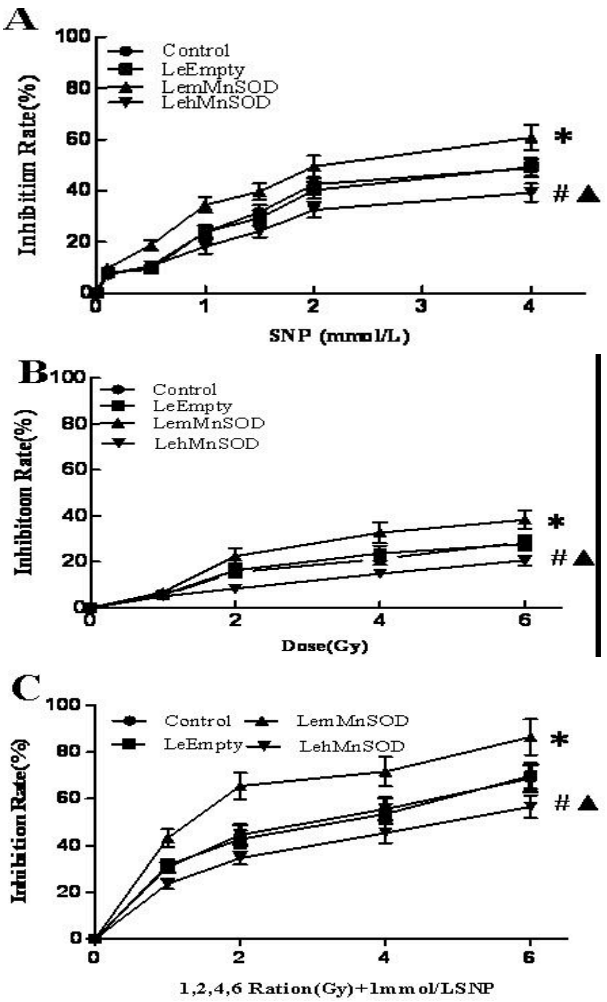

Figure 2. Effect of Manganese Superoxide Dismutase (MnSOD) Overexpression on the Proliferation of Cells Treated with Sodium Nitroprusside (SNP) and Radiation. Growth was inhibited in M-LeMnSOD cells compared with the two untransfected cell lines. Conversely, growth of H-LeMnSOD cells was promoted and was statistically different from that of the two untransfected cell lines or M-LeMnSOD cells. Data are means ± standard deviation (SD), n = 3. *$p < 0.05$ and #$p < 0.05$ compared to the two untransfected cell lines; ▲$p < 0.05$ compared to M-LeMnSOD cells. (A) 3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate cell survival after SNP treatment. Cells were seeded at 5000 cells/well in 96-well plates for 24 h and exposed to 0–4 mmol/L SNP for 48 h. B) MTT assay of radiation survival. Cells were seeded at 5000 cells/well in 96-well plates for 24 h and exposed to 0–6 Gy radiation, and then incubated for 48 h. (C) MTT assay of relative cell survival fraction. Cells were seeded at 5000 cells/well in 96-well plates for 24 h and exposed to 1 mmol/L SNP following treatment with a 2-Gy dose of radiation after 12 h, and then cultured for 48 h

by transfected moderate MnSOD cDNA (10.6 ± 1%), as indicated by Annexin V-FITC/PI staining, compared to the two untransfected MnSOD cell lines. Apoptosis in the control cells was similar to that of the LeEmpty cells (2.6 ± 0.2 and 2.5 ± 0.3%, respectively). Conversely, apoptosis was decreased in the H-LeMnSOD cells (1.0 ± 0.1%). During this process, gross intracellular ROS was detected using the DCFH-DA assay. Steady-state levels of intracellular ROS were notably decreased in M-LeMnSOD cells (MFI: 0.859 ± 0.040), compared to control cells

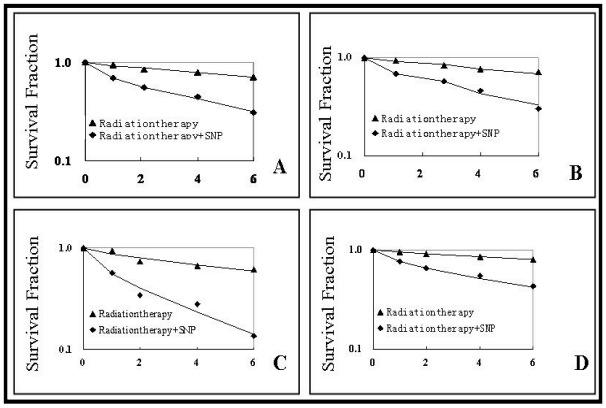

Figure 3. Multi-Target Single-hit Model-fitted Dose-Survival Curve of Cells. Cells were seeded at 5000 cells/well in 96-well plates for 24 h and exposed to 1 mmol/L sodium nitroprusside (SNP) following treatment with a 2-Gy dose of radiation after 12 h, and then cultured for 48 h. M-LeMnSOD cells exhibited increased radiosensitivity to SNP and radiation, but decreased radiosensitivity to nitric oxide and radiation. A, B, C, and D represent control, LeEmpty, M-LeMnSOD, and H-LeMnSOD cells, respectively

Table 1. Multi-target Single-hit Model Fitted Dose-survival Curve of Cells

Groups		D_0	N	Dq	SER
Control	Radiation	26.40	0.78	20.64	2.68
	Radiation+SNP	9.84	0.50	4.91	
LeEmpty	Radiation	24.85	0.77	19.09	2.50
	Radiation+SNP	9.94	0.50	5.01	
M-LeMnSOD	Radiation	19.27	0.69	13.24	3.90
	Radiation+SNP	4.95	0.46	2.28	
H-LeMnSOD	Radiation	31.27	0.90	28.20	2.08
	Radiation+SNP	15.04	0.52	7.87	

(MFI: 1.000 ± 0.042) and LeEmpty cells (MFI: 1.002 ± 0.047). However, there was also a significant decrease in the ROS levels of H-LeMnSOD cells (MFI: 0.763 ± 0.039) compared with the two untransfected MnSOD cell lines. Specifically, this may suggest a bidirectional effect of MnSOD overexpression on esophageal cancer cell proliferation in association with intracellular ROS levels.

Effects of MnSOD overexpression on cell proliferation combined with SNP or radiation

To further verify the biological effects of MnSOD overexpression on cell proliferation when combined with SNP or radiation, cells were incubated continuously with 0–4 mmol/L SNP for 48 h, or exposed to 0–6 Gy doses of radiation followed by incubation for 48 h. The results (Figure 2A) indicate an increase in the proliferation of H-LeMnSOD cells, which were resistant to SNP concentrations up to 0.5 mmol/L, indicating that high MnSOD overexpression correlates to NO resistance. On the contrary, M-LeMnSOD cells exhibited a decrease in cellular proliferation and the cells were significantly sensitive to NO at concentrations up to 0.5 mmol/L, suggesting that moderate MnSOD overexpression increases NO sensitivity. As in the SNP treatment, H-LeMnSOD cells were more radioresistant than the two untransfected cell lines when exposed to 2, 4, and 6 Gy radiation doses. M-LeMnSOD cells significantly

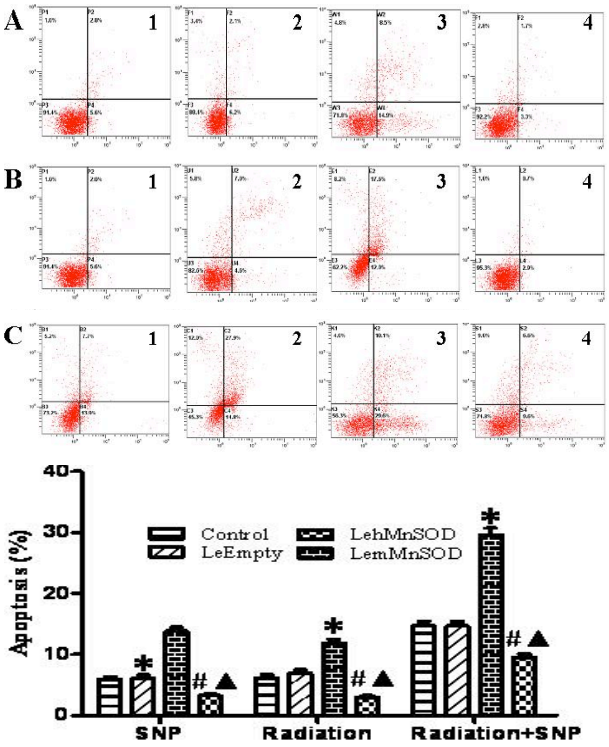

Figure 4. Apoptosis was Analyzed by Flow Cytometry Using Annexin V-FITC/PI Staining. Data are presented as mean values with standard deviation (SD; n = 3). *p < 0.05 and #p < 0.05 compared to the two untransfected cell lines; ▲p < 0.05 compared to M-LeMnSOD cells. (A), (B), and (C) represent cells treated with 1 mmol/L SNP, 2Gy radiation, and 1 mmol/L SNP + 2Gy radiation, respectively. 1, 2, 3, and 4 represent control cells, LeEmpty cells, M-LeMnSOD cells, and H-LeMnSOD cells, respectively

reversed radioresistance and increased radiosensitivity when compared to the two untransfected cell lines (Figure 2B).

In the next series of experiments (Figure 2C), we used 1 mmol/L SNP and a 2 Gy radiation dose to examine MnSOD overexpression sensitivity. M-LeMnSOD cell proliferation was enhanced and the cells were notably sensitized to SNP in combination with radiation, compared to the two untransfected cell lines. The growth of H-LeMnSOD cells was accelerated and the cells were notably radioresistant when treated with SNP and radiation. Moreover, in multi-target single-hit model-fitting survival curves, the values of D_0, Dq, N, and SER in M-LeMnSOD cell lines were less than in the two untransfected cell lines and in the H-LeMnSOD cells, indicating that M-LeMnSOD cells were more radioresistant and radiosensitive than H-LeMnSOD cells (Table 1, Figure 3). When M-LeMnSOD cells were treated with 1 mmol/L SNP (Figure 4A), apoptosis (13.8 ± 0.7%) was significantly higher than that of two the untransfected cell lines (6.0 ± 0.4, 6.2 ± 0.5%). Conversely, apoptosis was decreased in H-LeMnSOD cells (3.3 ± 0.3%). Moderate MnSOD protein expression was identified in M-LeMnSOD cells when apoptosis was highest (0.334 ± 0.029) compared to the protein expression in the two untransfected cell lines (0.215 ± 0.017, 0.193 ± 0.019, respectively). H-LeMnSOD cells, however, showed a large increase in protein expression (0.415 ± 0.038)

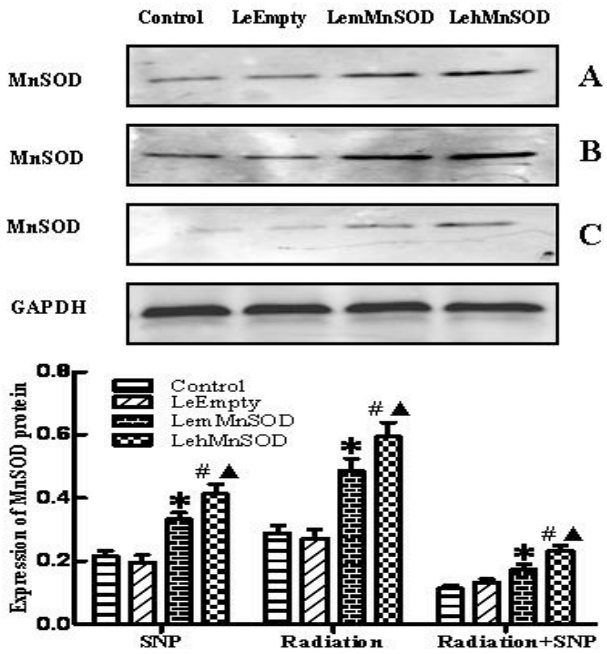

Figure 5. Sodium Nitroprusside (SNP) or/and Radiation-induced Manganese Superoxide Dismutase (MnSOD) Expression. Cells were treated with SNP (1 mmol/L) or radiation (2 Gy) for 48 h as indicated, and MnSOD was detected using a Western blot. The expression of MnSOD protein was observed in the ~26-kDa band. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are presented as mean values with standard deviation (SD; n = 3). *p < 0.05 and #p < 0.05 compared to the two untransfected cell lines; ▲p < 0.05 compared to M-LeMnSOD cells. (A), (B), and (C) represent cells treated with 1 mmol/L SNP, 2Gy radiation, and 1 mmol/L SNP + 2Gy radiation, respectively

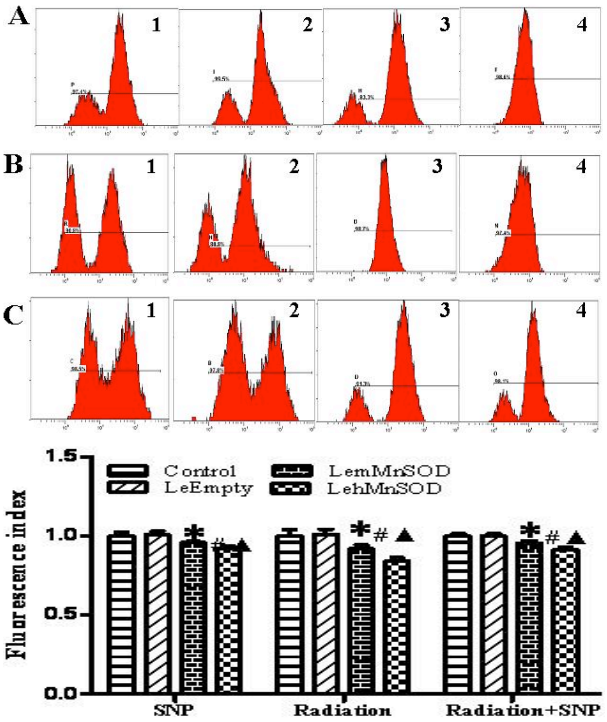

Figure 6. Steady-state Levels of Gross Reactive Oxygen Species (ROS) in TE-1 Cells as Determined by Flow Cytometry. Cells were seeded in 60-mm dishes for 24 h, treated with 1 mmol/L sodium nitroprusside (SNP) for 48 h; exposed to 2 Gy of radiation then incubated for 48 h; or exposed to 1 mmol/L SNP following treatment with a 2-Gy dose of radiation after 12 h followed by culture for 48 h. Monolayer cultures were harvested and trypsinized, washed once with phosphate buffered saline, and labeled for 30 min with 10 μM 2',7'-dichloro-fluorescin at 37ºC in a darkroom. Mean fluorescence intensity of 10,000 cells was analyzed using flow cytometry. Samples were assayed in triplicate; data are presented as means ± standard deviation of three separate experiments. *p < 0.05 and #p < 0.05 compared to the two untransfected cell lines; ▲p < 0.05 compared to M-LeMnSOD cells. (A), (B), and (C) represent groups treated with 1 mmol/L SNP, 2 Gy radiation and 1 mmol/L SNP +2 Gy radiation, respectively. 1, 2, 3, and 4 represent control, LeEmpty, M-LeMnSOD, and H-LeMnSOD cells, respectively

(Figure 5A).

To determine whether intracellular ROS levels were associated with SNP sensitivity and cell apoptosis, flow cytometry was used to detect intracellular ROS levels. Cells overexpressing MnSOD that were treated with 1 mmol/L SNP showed significant decreases in intracellular gross ROS (MFI: 0.956 ± 0.011), with significant decreases in ROS levels measured in H-LeMnSOD cells (MFI: 0.763 ± 0.039) compared with the two untransfected cell lines, in which no differences in MFI (1.000 ± 0.021, 1.004 ± 0.026) were found. These results provide evidence of decreased intracellular ROS modulated by MnSOD overexpression (Figure 6A).

We hypothesized that moderate MnSOD overexpression combined with moderate intracellular ROS would primarily kill cancer cells, but high MnSOD overexpression could protect normal cells from apoptosis. To determine whether this occurred, we analyzed MnSOD overexpressing cells irradiated with 2 Gy of radiation using flow cytometry and Western blot analysis. M-LeMnSOD cells had higher apoptotic levels compared with the two untransfected cell lines (11.97 ± 0.6 versus 7.2 ± 0.5 and 6.9 ± 0.6%), whereas the lowest level of apoptosis was measured in the H-LeMnSOD cells (3.0 ± 0.3%, Figure 4B). Moderate MnSOD protein expression (0.485 ± 0.040) was identified in M-LeMnSOD cells, which was higher than in the two untransfected cell lines (0.290 ± 0.026 and 0.273 ± 0.027) even though H-LeMnSOD cells showed the largest increase in protein expression (0.604 ± 0.059; Figure 5B). Intracellular ROS was significantly decreased in M-LeMnSOD cells treated with 2 Gy of radiation compared with the two untransfected cell lines (MFI: 0.917 ± 0.025 versus 1.000 ± 0.037 and 1.010 ± 0.031), but higher than that of H-LeMnSOD cells (0.838 ± 0.024; Figure 6B). Furthermore, treatment of M-LeMnSOD cells with 1 mmol/L SNP and 2 Gy radiation had a notable effect on SNP and radiation-induced apoptosis (29.6 ± 1.1%), whereas the same conditions applied to H-LeMnSOD cells reversed apoptosis (9.5 ± 0.6%) compared to the control cells (14.7 ± 0.8%) and the LeEmpty cells (14.7 ± 0.7%, Figure 4C). This was further confirmed by Western blot analysis (Figure 5C). These results suggest a link between the effects of varying MnSOD levels and the regulation of ROS produced by the different treatment protocols.

To confirm the regulation of ROS by MnSOD, we identified the intracellular ROS levels induced by 1 mmol/L SNP and 2 Gy of radiation. Similar decreases in intracellular ROS were measured in M-LeMnSOD (MFI:

0.953 ± 0.017) and H-LeMnSOD cells (MFI: 0.913 ± 0.013) compared with the two untransfected cell lines (MFI: 1.000 ± 0.012 and 0.998 ± 0.016, respectively, Figure 6C). Collectively, these data indicate that moderate MnSOD overexpression, but not high overexpression, sensitizes esophageal cancer cells to ROS.

Discussion

Esophageal cancer is the sixth leading cause of death from cancer (Enzinger and Mayer, 2003). Although recent advances have contributed to the improvement of diagnosis, treatment and prognosis of this disease, treatment strategies are still needed due to chemotherapy-induced tumor resistance. Among various therapies, radiation therapy has been used extensively in the management of patients with cancer of the esophagus. Radiation therapy induces cytotoxicity through a progression of molecular pathways that leads to the production of ROS (Biaglow et al., 1992). Nevertheless, the underlying mechanism of radiotherapy as well as the resistance to radiotherapy is not fully understood.

The antioxidant enzyme MnSOD, which is localized almost exclusively in the mitochondria, catalyzes the dismutation of the superoxide anions into hydrogen peroxide (Wispe et al., 1989). Overexpression of MnSOD through the use of minicircle DNA containing the human MnSOD transgene has been shown to protect against ionizing irradiation both in vitro and in vivo (Zhang et al., 2008). In addition, up-regulation of MnSOD expression enhanced the radiosensitivity in rat fibrosarcoma cells (Urano et al., 1995). MnSOD overexpression also produced radioresistance when the plasmid containing MnSOD gene was transfected into caner cells, which was reversed by the transfection of antisense MnSOD cDNA (Epperly et al., 2003). Moreover, silencing of MnSOD by microRNA interference enhanced the radiosensitivity of nasopharyngeal carcinoma cells to ionizing radiation injury (Qu et al., 2010; Qu et al., 2010). A previous study demonstrated that PE and cellular adhesion properties changed bidirectionally according to the levels of MnSOD overexpression, first increasing then decreasing as MnSOD activity increased in SaOS2, a human osteosarcoma cell line (Komatsu et al., 2005).

We have shown previously that reduced levels of MnSOD mRNA and protein were observed in esophageal squamous cell carcinoma tissue compared to that of normal esophageal tissue (Sun et al., 2011). Hence, in this present study, we investigated the potential role of MnSOD overexpression in the radiosensitivity and radioresistance of esophageal squamous carcinoma by transfecting the MnSOD gene into human esophageal squamous carcinoma TE-1 cells (Nishihira et al., 1979). Our results demonstrated that the expression level of MnSOD in TE-1 cells was critical in determining the outcomes of radiation therapy since overexpression of MnSOD at high levels was resistant to radiation, whereas moderate overexpression of MnSOD led to radiosensitivity.

Varying proportions of superoxide radicals and hydrogen peroxide have different effects on cytotoxicity, and may explain the bidirectional effects of MnSOD, thus providing a reasonable interpretation of our results (Wang et al., 2005). As intraesophageal administration of MnSOD yields radioprotection in esophageal cells (Niu et al., 2010), we suggested that the expression level of MnSOD should be carefully determined to selectively induce tumor cell damage without affecting normal esophageal cells.

NO, produced from L-arginine by the catalysis of nitric oxide synthase (NOS), serves as a reactive free radical in multiple biological and physiological processes (Nathan et al., 1992). Low concentrations of NO have been found to enhance tumor growth, whereas overproduction of NO can induce apoptosis through unknown mechanisms in neural cells, macrophages, cancer cells, and other cell types (Sang et al., 2010). NO administration has been shown to increase the radiosensitivity of mouse mammary carcinoma EMT-6 cells (Janssens et al., 1999), Chinese hamster V79 cells (Pavord et al., 1996), and sarcomatous SCK cells (Lindsell and Griffin, 1999). NO interacts with DNA and leads to single-stranded DNA breaks, which enhances MnSOD-induced tumor-growth inhibition. In addition, mitochondria is the main source of ROS generation, and also plays an important role in cell apoptosis (Ott et al., 2007) (Orrenius et al., 2007). Nevertheless, few studies have revealed the association between MnSOD overexpression and NO radiosensitivity.

Komatsu et al. (2005) found that NO loading increased hydrogen peroxide levels that resulted from the overexpression of MnSOD, which synergistically reduced PE and cellular adhesion in SaOS2, a human osteosarcoma cell line. Wang et al. showed that the overexpression of MnSOD was accompanied by an increase in NO levels, and could either inhibit or improve adriamycin sensitivity in human osteosarcoma cells (Wang et al., 2005). In this study, we determined that the overexpression of MnSOD influenced the effects of NO and radiation on esophageal cancer cells both in a positive and negative manner. We found that moderate MnSOD overexpression inhibited proliferation and increased tumor cell apoptosis at various radiation dosages. Notably, when the NO concentration was higher than 1.0 mmol/L and radiation was greater than 2 Gy, growth inhibition was induced by moderate overexpression of MnSOD, which greatly increased in a concentration-and dose-dependent manner. These observations were in accordance with the growth inhibition induced by NO detected in the gastric cancer cell lines, AGS (Sang et al., 2010) and BGC-823 (Lin et al., 2001). However, overexpression of MnSOD at relatively high levels combined with NO and radiation accelerated cell proliferation and reduced apoptosis. The reason for this discrepancy remains unclear. It is possible that MnSOD may be assuming different roles, which changes with different expression levels. Also, the varied proportions of ROS may be affecting the production of apoptotic proteins. Further investigation is needed to elucidate the mechanisms of these effects.

TE-1 cells with moderate MnSOD overexpression were more radiosensitive in the presence of NO, whereas higher MnSOD overexpression increased radioresistance. These observations raised the question of whether the radiosensitivity or radioresistance generated by different levels of MnSOD overexpression could protect normal

cells. Nonetheless, the underlying mechanism of such phenomenon is not yet fully understood. We discovered that the elevated sensitivity was not strictly concentration- or dose-dependent, but could be maximized. We speculated that the combination of chemotherapy agents and radiation might produce different types and quantities of ROS, and initiate the activation of different apoptotic pathways, than chemotherapy or radiation treatments alone. Wang et al. (2005) demonstrated that $SaOS_2$ cells were sensitized to ADR by different levels of MnSOD overexpression in a bidirectional pattern, with high overexpression reducing radiosensitivity by promoting apoptotic cell death and low overexpression increasing radiosensitivity by suppressing cell apoptosis. We hypothesized that this was due to different proportions of ROS that were generated, which induced different reaction products.

Furthermore, in this present study, we found that the MFI of ROS was highest in untransfected MnSOD cell lines and lowest in cells with high MnSOD overexpression, while cells with moderate MnSOD overexpression had intermediate MFI values. Lau et al. (2004) reported that highly concentrated ROS produced by nitrites could induce the apoptosis of pulmonary epithelial cells (LEC), which could be prevented by administration of glutathione. Nitrites produced chronic, low concentrations of ROS and transformed LEC into malignant tumors (Lau and Chiu, 2006), suggesting that the proliferative potential and differentiation of the tumor cells could be determined solely by the ROS content (Lau et al., 2008).

In summary, our present study showed that the expression level of MnSOD was of great importance for regulating the radioresistance and radiosensitivity of esophageal cancer cells. Moderate overexpression of MnSOD increased the radiosensitivity of esophageal cancer cells, whereas high MnSOD overexpression had the opposite effect on cell radiosensitivity. The regulatory role of MnSOD overexpression on radioresistance and radiosensitivity of tumor cells represented an intriguing new method to kill tumor cells that could also be used to protect normal cells from radiation damage. However, previous studies revealed that overexpression of MnSOD protected leukemia exposed in vitro to ionizing radiation, whereas it did not provide radioprotective effects to tumor cells exposed to ionizing irradiation in vivo (Weiss et al., 1993). Therefore, future investigations will be needed to elucidate the potential regulatory role of MnSOD expression on radioresistance and radiosensitivity of esophageal squamous carcinoma cells in vivo.

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