Published in final edited form as:

Radiat Res. 2013 August ; 180(2): 189–204. doi:10.1667/RR3177.1.

Conditional Radioresistance of tet-Inducible Manganese Superoxide Dismutase Bone Marrow Stromal Cell Lines

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Abstract

Mitochondrial targeted manganese superoxide dismutase is a major antioxidant enzyme, the levels of which modulate the response of cells, tissues and organs to ionizing irradiation. We developed a Tet-regulated MnSOD mouse (MnSOD^{tet}) to examine the detailed relationship between cellular MnSOD concentration and radioresistance and carried out *in vitro* studies using bone marrow culture derived stromal cell lines (mesenchymal stem cells). Homozygous MnSOD^{tet/tet} cells had low levels of MnSOD, reduced viability and proliferation, increased radiosensitivity, elevated overall antioxidant stores, and defects in cell proliferation and DNA strand-break repair. Doxycycline (doxy) treatment of MnSOD^{tet/tet} cells increased MnSOD levels and radioresistance from $\bar{n}$ of 2.79 ± 1.04 to 8.69 ± 1.09 ($P = 0.0060$) and normalized other biologic parameters. In contrast, MnSOD^{tet/tet} cells showed minimal difference in baseline and radiation induced mRNA and protein levels of TGF- β , Nrf2 and NF- κ B and radiation induced cell cycle arrest was not dependent upon MnSOD level. These novel MnSOD^{tet/tet} mouse derived cells should be valuable for elucidating several parameters of the oxidative stress response to ionizing radiation.

INTRODUCTION

Much evidence supports a critical role of oxidative stress in the acute response of cells, tissues and organs to ionizing radiation (1–6). Radiation resistance of cells in culture has been correlated with the level of antioxidant stores in the mitochondria (6). The cellular radiation damage response has been linked to activation of both redox sensitive (Nrf2) (7–9) and DNA strand-break dependent (NF- κ B) (3) promoter binding proteins that regulate inflammatory (6, 8–12), and cytokine response factors including TGF- β , IL-1, TNF- α and IFN- γ (13–18).

The cellular ionizing radiation response is mediated in part by small molecule antioxidants including glutathione (6, 19) and the enzymes manganese superoxide dismutase (MnSOD), catalase and glutathione peroxidase (2, 5, 19). Depletion of one or both categories of cellular antioxidant stores can increase the magnitude of acute radiation damage (2–3, 6, 19).

MnSOD is a prominent first line of defense against radiation damage (6, 20–24). MnSOD is also involved in stabilization of cellular genetic (4–5) and metabolic (20–22) aspects of tissue and organ physiology. Overexpression of MnSOD *in vivo* (25) decreases both acute radiation damage and late radiation fibrosis (15). Stably increased or decreased levels of MnSOD in transgenic overexpressing (26) or null (27) mouse models, respectively, have been reported and transient acute increase in MnSOD overexpression by transgene transfection increases normal tissue radioresistance (28–31).

To gain further insight into the effect of regulated MnSOD levels on cell and tissue radiobiology, we have developed a novel conditional MnSOD^{tet/tet} allele, in which endogenous MnSOD expression is inducible by a Tet response element in its promoter (32–35). Bone marrow stromal cell lines derived from MnSOD^{tet/tet} mice revealed that induced levels of MnSOD expression correlated with reversible changes in several biological and biochemical parameters including: radiosensitivity in clonogenic survival curves, viability, cell doubling, DNA strand-break repair and overall antioxidant level.

MATERIALS AND METHODS

Tet-On MnSOD Allele Construction

The *MnSOD*^{tet} mutant allele was generated through targeted mutagenesis of the endogenous *Sod2* (*MnSOD*) allele in TC1 mouse ES cells. The gene-targeting vector was constructed from a plasmid containing the endogenous 129/Sv wild-type *MnSOD* allele. A 5.3-kb tetracycline (Tet-On) gene regulatory fragment was inserted into a *SwaI* restriction site located approximately 30 nucleotides 5' of the *MnSOD* initiation codon in the first *MnSOD* exon. The Tet-On regulatory fragment is a modification of the *neo^r* version of the Tet-Off regulatory cassette previously used (32–35). The Tet-Off cassette (in pBluescript) was converted to a Tet-On cassette by changing five codons by site-directed mutagenesis (Stratagene QuickChange KitTM). The codon changes are: S12G(ggc), E19G(ggg), A56P(ccc), D148E(gag) and H179R(cgc). These amino acid changes converted tTA to the M2 form of rtTA (rtTA-M2). The 5.3-kb Tet-On fragment was removed from the pBluescript vector by digestion with *SpeI* and *NotI*, filled in and cloned into the *SwaI* restriction site of the *MnSOD* plasmid to generate the *MnSOD*^{tet-on} targeting plasmid. This plasmid was linearized by digestion with *NotI*, electroporated into TC1 ES cells and heterozygous G418-resistant *MnSOD*^{tet/+} ES colonies identified by Southern-blot screening. Cells from one *MnSOD*^{tet/+} colony were injected into C57BL/6 blastocysts to generate a *MnSOD*^{tet} mouse line, which has been maintained in a mixed C57BL/6–129/Sv strain background.

MnSOD^{tet} ES cells and mice were genotyped by Southern blotting or by PCR. Southern blots of *EcoRI*-digested tail DNA were hybridized with a 5' DNA probe constructed by PCR amplification of mouse genomic DNA with oligonucleotides 5' AAG GAG TGA CAG GGC AGA TG 3' and 5' CCT TAA GGG GCA GGC TAT TC 3'. The probe hybridized to a 24-kb wild-type *MnSOD* genomic fragment and a 12.8-kb *MnSOD*^{tet} fragment (Fig. 1). Conditions for genotyping by PCR were 94°C for 10 min; 35 cycles of 94°C for 45 s; 58°C for 45 s; 72°C for 1 min; 72°C for 10 min. The wild-type *MnSOD* allele yielded a 473-bp PCR product using oligonucleotides MnSODwtR (5' CAT GAT CTG CGG GTT AAT GT 3') and MnSODwtF (5' AAT TTG GCA CAG GGG AGA C 3'). The *MnSOD*^{tet} allele yielded a 281-bp PCR product using oligonucleotides MnSODwtF and MnSODTetR (5' CAA ATC CTC CTC GTT TTT GG 3') (Fig. 1, see arrows).

Bone Marrow Stromal Cell Lines

In the absence of doxycycline, newborn MnSOD^{tet/tet} mice died within 72 h of birth, similar to MnSOD^{-/-} knockout mice (null) (21, 22). We established bone marrow cell lines from the adherent layer of continuous bone marrow cultures from newborn female mice, each of several genotypes: MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{+/+} mice using tissue culture techniques as described previously (36). Briefly, the contents of each newborn mouse femur and tibia were flushed using a 21-gauge needle and 25 cc syringe into a T25 flask in 15 ml of Fisher's medium supplemented with 15% heat activated fetal bovine serum (37). Permanent cell lines were derived from passaged cells from the adherent cell layer. Clonal lines were established from the passaged adherent cell layer according to published methods (37). Tail tissue was used to genotype the mice as described in the *Tet-On MnSOD Allele Construction* section. Cell lines were cultured in 21% oxygen in a high-humidity atmosphere. A previously described, MnSOD^{-/-} (MnSOD-null) embryo fibroblast cell line was used as a control (27). All adherent cell lines were passaged weekly by a 1:5 split for 4 weeks, then passaged at greater dilutions of 1:10 and 1:100 weekly for one year. There was no evidence of cellular senescence during maximum duration (12 months) of the present experiments.

Immunohistochemistry for MnSOD Protein

For immunofluorescence staining for intracellular MnSOD in the MnSOD^{+/+} wild-type (C57BL/6 background strain) and MnSOD^{tet/tet} bone marrow stromal cell lines, cells were seeded onto polylysine coated glass cover slips (BD Biosciences, Franklin Lakes, NJ). Paraformaldehyde-fixed cells were stained with a rabbit polyclonal primary antibody against superoxide dismutase 2 (Abcam, Cambridge, MA) and AlexaFluor 488 secondary antibody (Invitrogen, Gaithersburg, MD). AlexaFluor 568 phalloidin was used to stain F actin (Invitrogen, Gaithersburg, MD). Nuclei were counterstained with DAPI (Invitrogen, Gaithersburg, MD).

Western Analysis for MnSOD, TGF- β , Nrf2 and NF- κ B Protein Expression

To establish the level of MnSOD in MnSOD^{tet/tet} cells, harvested cells from each of several cell lines were harvested and lysed in NP-400 buffer [50 mM Tris, pH 7.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP-40 and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN)]. Protein samples were separated in 15% polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. Primary anti-MnSOD antibody (Novus Biologicals, Littleton, CO) or controls including B-actin or GAPDH (Sigma Aldrich, St. Louis, MO) antibody were used. Horseradish peroxidase anti-rabbit or anti-mouse secondary antibody (Promega, Pittsburgh, PA) was then applied and membranes developed with Super Signal West Dura ECL (Thermo Scientific, Rockford, IL). Antibodies against TGF- β , NF- κ B and Nrf2 were obtained from Santa Cruz Biochemical Laboratories, Santa Cruz, CA. For quantitation of levels of TGF- β , Nrf2 and NF- κ B, band densities were quantified with ImageJ, National Institutes of Health (www.rsweb.nih.gov/ij).

Induction of MnSOD by Doxycycline

To demonstrate induced MnSOD expression in MnSOD^{tet/tet} cells, doxycycline (doxy) (0.25 μ g/ml) was added to cultures of MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{+/+} cells and MnSOD^{-/-} null cells (27). Cells from each line were harvested after times in doxy ranging from 0 to 48 h, RNA extracted and RT-PCR for the MnSOD mRNA performed as described above. To determine the effect of doxy removal on reduced levels of expression of MnSOD mRNA, doxy was removed from cell cultures, which had been incubated previously in doxy

for 24 h. Cells were harvested at times ranging from 0–24 h after removal of doxy, RNA isolated and RT-PCR for the MnSOD transcript was performed as described previously (37).

Measurement of MnSOD Biochemical Activity

To demonstrate that doxy treatment altered MnSOD biochemical activity, cells from each cell line: MnSOD^{tet/tet}, MnSOD^{tet/+}, MnSOD^{+/+} and MnSOD^{-/-} were incubated in the presence or absence of doxy (0.25 µg/ml) for 24 h. The cells were then harvested and analyzed for MnSOD biochemical activity as previously described (24). Briefly, the cells were lysed by three cycles of rapidly freezing and thawing followed by sonication. Protein concentration were then determined and concentrations ranging from 0–200 µg protein in 100 µl volume of TBS were added to 900 µl of MnSOD activity buffer (1.8 mM Xanthine, 0.33 M NaCN, 2.24 mM NBT, 1.33 mM DETAPAC, 40 U/ml catalase, 10 mg/ml BSA and 10 mM BCS (bathocuprine disulfonic acid) and 100 µl placed in each well of a 96-well plate. The MnSOD biochemical activity was determined by adding 10⁻² U of xanthine oxidase to each well. The plate was immediately placed in a plate reader and the OD was measured at 560 nm every minute for 5 min. One unit of MnSOD biochemical activity resulted in a 50% reduction of the color change resulting from the reduction of NBT by superoxides produced from the reaction of xanthine with xanthine oxidase.

In Vitro Clonogenic Radiation Survival Curves

Cells from each cell line were suspended at 1 × 10⁶ cells/ml and irradiated in suspension to doses of 0 to 8 Gy using a Shepherd Mark 1 irradiator with a cesium source (J. L. Shepherd, San Fernando, CA), dose rate 70 cGy/min. Cells were plated in 4-well Linbro[®] tissue culture plates (MP Biomedicals, LLC, Salon, OH) as described previously (27, 37) and incubated at 37°C, 21% oxygen, with 5% CO₂ for 7–14 days, stained with crystal violet and colonies of greater than 50 cells were counted using a GelCount colony counter (Oxford Optronix, Oxford, UK). The *in vitro* radiation survival curves were analyzed by both linear-quadratic model and the single-hit multitarget model, and were compared using the final slope representing multiple-event killing (D₀) and the extrapolation number measuring the width of the shoulder on the radiation survival curve ($\bar{n}$) (19). Results for D₀ and $\bar{n}$ are presented as the mean ± standard error of the mean (SEM) from multiple (at least 3) measurements. The two-sided two-sample *t* test was used to compare means of different groups.

Effect of Doxy on the Radiosensitivity of Plateau Phase MnSOD^{tet/tet} Cells In Vitro

Cells from MnSOD^{tet/tet}, MnSOD^{+/-}, MnSOD^{+/+} and MnSOD^{-/-} lines were grown to confluence and doxy (0.25 µg/ml) was added to half of the cultures. Twenty-four hours later the cells were irradiated to doses ranging from 0–10 Gy. Every 48 h media was changed to maintain doxy concentration at 0.25 µg/ml. At days 7 and 14 after irradiation, cells were trypanized and both the viability and number of cells determined using a Vi-Cell XR (Beckman Coulter, Inc., Indianapolis, IN). The viability and total cell number was reported as mean ± standard deviation for each subgroup, plotted on a graph with a line drawn connecting the points. The slope of the line was determined using a linear mixed model. The F test in this model was used to compare slopes (i.e., the increase in viability for every 1 Gy increase in radiation dose) between any two groups. A significant *P* value indicates a significant difference in the dose effect between two groups (42, 43).

Effect of Radiation on Induction of RNA Transcripts Associated with the Acute Radiation Response in MnSOD^{tet/tet} Cells

Real-time polymerase chain reaction (RT-PCR) analysis for radiation inducible transcripts for MnSOD, TGF- β , Nrf2, and NF- κ B was performed as described previously (37). Cells

from each cell line (MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} and MnSOD^{-/-}) were irradiated in exponential growth phase to 10 Gy and cells harvested at 0, 1 or 6 h after irradiation. RNA was extracted and cDNA synthesized for quantitation of levels of expression using primers specific for the promoter regions of MnSOD, TGF- β , Nrf2 and NF- κ B using RT-PCR (37). The results were presented as percentage fold increases in RNA above baseline levels of nonirradiated C57BL/6NHsd wild-type mouse bone marrow stromal cell line. These baseline levels allowed us to determine the magnitude of elevation in RNA detectable by robot RT-PCR that was attributable to doxy treatment and/or irradiation of each cell line. The MnSOD^{+/+} bone marrow stromal cell line derived from the background mouse strain used to derive the MnSOD^{tet/tet} mouse was indistinguishable in RT-PCR responses from the C57BL/6NHsd mouse long-term marrow culture derived stromal cell line used as a control for these experiments. For each gene (MnSOD, TGF- β , Nrf2 or NF- κ B), expression was analyzed with three-way ANOVA where cell type (MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} or MnSOD^{-/-}) treatment (doxy or none), time after treatment (0, 1 or 6 h) and interactions were used as explanatory factors. Post hoc pairwise comparisons were done by F tests using CONTRAST statement in SAS Proc GLM. For these comparisons, *P* values less than 0.05 were regarded as significant. As an exploratory study, *P* values were not adjusted for multiple comparisons.

Assay for Intracellular Antioxidant Levels

MnSOD^{+/+}, MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{-/-} cells were incubated in 0.25 μ g/ml doxy for 24 h and then irradiated to 10 Gy. Cells were harvested at each of 3 time points: immediately after irradiation, at 4 or 24 h after irradiation and snap-frozen in liquid nitrogen. Cell pellets were then thawed and mechanically homogenized in cold phosphate buffer solution. Protein concentrations were standardized by Bradford assay and antioxidant reductive capacity (antioxidant status) was measured using a commercial kit (Northwest Life Science Specialties, Vancouver, WA). This assay measures the antioxidant capacity of cells based on the ability of cellular antioxidants to reduce Cu⁺⁺ to Cu⁺ which reacts with bathocuproine to form a color complex with absorbance at 480–490 nm. The antioxidant activity was compared to a standard curve generated using trolox units (milliequivalents) and all data was, therefore, expressed as trolox units.

Measurement of Radiation induced DNA Strand Breaks by Comet Assay

The magnitude of DNA strand breaks at serial time points after irradiation of each cell line were determined using a neutral comet tail-length assay (CometAssay, Trevigen, Gaithersburg, MD). Cells from each cell line including: MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} and MnSOD^{-/-} were irradiated to 5 Gy. At each of several time points after irradiation including preirradiation (0), or at 10, 20 or 30 min, cells were placed on ice for 30 min after which time they were mixed with agarose and placed on CometSlides. RNA and protein were digested and the DNA electrophoresized. Comet tail moment was measured and the data presented as comet tail moment reported as the mean $\pm$ standard deviation (SD) for each culture cell line and time subgroup. Each subgroup was defined by cell line, presence or absence of doxy treatment, irradiation dose and time of measurement (pre-, 10, 20 or 30 min after irradiation). The data were graphed with a line drawn connecting the individual data points, which was log-transformed to be normally distributed based on the Shapiro-Wilk normality test (43). Two-sample *t* tests were used to compare the logarithm transformed tail intensity between any two groups. A linear mixed model was then built on the log-transformed data in each of the 5 Gy irradiated groups, using group and time of measurement. The interaction term of each line and condition was reported as a fixed explanatory variable, and the experiment ID was used as a random effect (a total of 9 experiments were performed). Each group was defined as the combination of cell line [MnSOD^{+/+}, MnSOD^{tet/tet}, MnSOD^{tet/+} or MnSOD^{-/-} (21, 22) cells] radiation dose and

drug treatment (with or without doxycycline). The F test in this model was used to compare slopes between any two groups. A significant *P* value indicates a significant difference in the change in intensity with time. A *P* value of less than 0.05 was interpreted as a significant difference. As an explanatory analysis, *P* values were not adjusted for multiple comparisons. These analyses were carried out with SAS software.

RESULTS

Construction of MnSOD^{tet/tet} Mice and Documentation of Genotype

As shown in the Materials and Methods section and Fig. 1, the wild-type *MnSOD* (*SOD2*) allele yielded a 473 basepair (bp) PCR product and the MnSOD^{tet} allele yielded a 281 bp PCR product. Genotyping was achieved by analyzing DNA from the tip of the tail of one-day-old newborn mice. MnSOD^{tet/tet} mice demonstrated only the MnSOD^{tet} allele product of 281 basepairs. In contrast, the larger 473 bp allele was detected in wild-type MnSOD^{+/+} or MnSOD^{tet/+} mice.

Bone Marrow Stromal Cell Lines Derived from MnSOD^{tet/tet} Mice Demonstrate Distinct Doxycycline Inducible Biologic Properties

We established cell lines from the adherent layer of long-term bone marrow cultures of individual genotyped newborn mice derived from breeding MnSOD^{tet/+} mice. Clonal bone marrow stromal cell lines from the adherent layer of MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{+/+} bone marrow cultures were then established. Western analysis for the MnSOD protein demonstrated MnSOD^{-/-} cells has none and MnSOD^{tet/tet} and MnSOD^{tet/+} cells had decreased levels of MnSOD protein compared to the MnSOD^{+/+} cell line (Fig. 2). Quantitation of the bands demonstrated that MnSOD^{+/+} cells had a 1.8 ± 0.1 -fold ($P = 0.0125$) and 55.1 ± 2.5 fold ($P = 0.0022$) increase in level of MnSOD expression compared to MnSOD^{tet/+} and MnSOD^{tet/tet} cells, respectively. To demonstrate that incubation of MnSOD^{tet/tet} cells in doxy increased levels of MnSOD, cells from each cell line were incubated in 0.25 µg/ml doxy and harvested at various times ranging from 4–48 h. MnSOD RNA was increased in MnSOD^{tet/tet} cells that were incubated in doxy ($P = 0.0135$) (Fig. 3A). To determine the time course of doxy induction of MnSOD, RNA was extracted from MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{+/+} cell lines at each of several times after incubation in doxy and RT-PCR carried out. The results demonstrated that increased MnSOD RNA was detected as early as 4 h and clearly at 12 h after addition of doxy (Fig. 3A). To determine the effect of removal of doxy on MnSOD levels in MnSOD^{tet/tet} cells, cells were incubated in the presence of doxy for 24 h and then the media was exchanged with fresh media containing no doxy. A clear decrease in MnSOD mRNA expression was detected within 1 h after removal of doxy (Fig. 3B). These results establish that MnSOD RNA levels in MnSOD^{tet/tet} cells were conditionally increased based upon the presence of doxy.

To quantitate biochemical levels of MnSOD in MnSOD^{tet/tet} cells relative to doxy treatment and the effect of doxy itself on the assay, we measured biochemical activity of MnSOD in doxy treated or control MnSOD^{tet/tet}, MnSOD^{tet/+}, MnSOD^{+/+} and MnSOD^{+/+} cells. In the absence of doxy MnSOD^{tet/tet} cells showed 3.7 ± 0.9 units and MnSOD^{tet/+} cells showed 7.2 ± 1.5 units, respectively, of biochemical MnSOD units per mg protein. Both were significantly lower than the level of 19.4 ± 3.4 ($P = 0.0108$ and $P = 0.0297$) measured in MnSOD^{+/+} cells (Fig. 4). MnSOD^{tet/tet} cells cultured in the absence of doxy had low but detectable MnSOD biochemical activity compared to the MnSOD^{-/-} unconditional knockout cell line (<1.0) (Fig. 4). These results are consistent with low-level leakage of TET-On controlled gene expression (35). Doxy treatment of MnSOD^{tet/+} and MnSOD^{tet/tet} cells in doxycycline for 24 h resulted in significantly increased MnSOD biochemical activity similar to the levels found in MnSOD^{+/+} cells (Fig. 4). The data confirm and extend other

experimental data obtained with Western blot and PCR analysis data, that showed low-background levels of MnSOD protein and RNA in MnSOD^{tet/tet} cells in the absence of doxy (Figs. 2 and 3, respectively). The low level of MnSOD biochemical activity and MnSOD protein in the MnSOD^{tet/tet} cells may have been attributable to low-level leakage of transcription and translation as reported with other tet inducible cell lines (35). There was no detectable doxy induction of MnSOD in MnSOD^{-/-} unconditional knockout cells or significant change in the level of MnSOD in MnSOD^{-/-} cells (<1 and 13.7 ± 3.5 units) (Fig. 4). The data establish that doxy induced significant increases in MnSOD biochemical activity in MnSOD^{tet/tet} and MnSOD^{tet/+} cell lines (11.1 ± 1.1 and 14.1 ± 0.9 units, respectively). ($P = 0.0135$ and 0.0405 , respectively)

We next determined the intracellular location of MnSOD protein in MnSOD^{tet/tet} cells. Immunohistochemistry demonstrated increased cytoplasmic MnSOD protein expression after doxy treatment of MnSOD^{tet/tet} but not MnSOD^{+/+} cells (Fig. 5). Cells from MnSOD^{tet/tet} and MnSOD^{+/+} were grown on cover slips in the presence or absence of doxy ($0.25 \mu\text{g/ml}$) for 24 h. The cells were then stained with a rabbit polyclonal antibody to MnSOD and an AlexaFluor 488 secondary antibody. No MnSOD protein was detected by immunohistochemistry in the MnSOD^{tet/tet} cell line in the absence of doxy (Fig. 5A and B), but these cells incubated in the presence of doxy demonstrated induced MnSOD protein (Fig. 5C and D). Unchanged levels of MnSOD protein were visualized in MnSOD^{+/+} cells grown in the absence or presence of doxy (Fig. 5E, F, G H, respectively).

Doxy Dependent Radiation Resistance of MnSOD^{tet/tet} Bone Marrow Stromal Cells

We next measured the effect of doxy induced MnSOD on the radiosensitivity of MnSOD^{tet/tet} bone marrow stromal cells by both clonogenic radiation survival curve assay and cell survival in confluent (plateau phase) cells. We first tested the radiation sensitivity of each cell line by clonogenic radiation survival curves. MnSOD^{tet/tet} compared to MnSOD^{+/+} and MnSOD^{tet/+} cells showed clear differences in radiosensitivity. There was a significant increase in the $\tilde{n}$ of MnSOD^{tet/tet} cells irradiated and grown in the presence of doxy compared to no doxy ($\tilde{n} = 8.69 \pm 1.09$ and $\tilde{n} = 2.79 \pm 1.04$, respectively, $P = 0.0060$) (Fig. 6). In contrast, there was no significant change in the clonogenic radiation survival curve of MnSOD^{+/+} or MnSOD^{tet/+} cells grown in the presence or absence of doxy. These data establish the doxy dependent radiosensitivity of MnSOD^{tet/tet} cells.

Cells from each cell line in confluence (plateau phase) were next tested for response to ionizing radiation. Cells grown in the presence or absence of doxy for 7 or 14 days after irradiation to 0, 2, 4, 6, 8 or 10 Gy showed a clear doxy dependent increase in survival of irradiated confluent MnSOD^{tet/tet} cells in culture (Tables 1 and 2). The data was plotted and the slopes of the line determined using a linear mixed model (42). At 14 days MnSOD^{tet/tet} cell lines had significantly decreased slopes for viability and marginally significant decrease in the total number of cells compared to MnSOD^{+/+} cells ($P = 0.0011$ or <0.0001 , respectively) indicating increased radiosensitivity of the MnSOD^{tet/tet} cells. Comparison of the viability of irradiated MnSOD^{+/+} and MnSOD^{tet/tet} cells grown in doxycycline showed no differences. This result establishes that the doxy induced increase in MnSOD expression returned the radioresistance of MnSOD^{tet/tet} plateau phase culture cells to control levels. Comparisons of the viability of irradiated MnSOD^{tet/tet} cells incubated in the presence of doxy at days 7 and 14 with cells grown without doxy showed an increase both days ($P = 0.0475$ and $P < 0.0001$, respectively) and in total cell number at day 7 ($P = 0.0266$).

Doxy Treatment does not Change the Cell Cycle Distribution of Irradiated MnSOD^{tet/tet} Cells

Cell cycle analysis of irradiated MnSOD^{tet/tet} cells revealed no doxy dependent differences from MnSOD^{+/+} cells in the radiation response at 24 h after 10 Gy (Fig. 7). Both MnSOD^{+/+} and MnSOD^{tet/tet} cells grown in the presence or absence of doxy, showed a significant increase in G₂M phase cells at 10 Gy compared to 0 Gy and a significant decrease in S phase cells (Fig. 7). There was no statistically significant difference between MnSOD^{tet/tet} cells in the presence or absence of doxy and MnSOD^{+/+} cells, (see Supplementary Table S6; <http://dx.doi.org/10.1667/RR3177.1.S1>). Thus, the doxy induced increase in level of MnSOD in MnSOD^{tet/tet} cells did not have a detectable effect on altering cell cycle distribution 24 h after irradiation.

Effect of Doxy on Antioxidant Levels in Irradiated MnSOD^{tet/tet} Cells

We next determined the total antioxidant status of the MnSOD^{tet/tet} cell line at 3 time points after 10 Gy irradiation in the presence or absence of doxy (immediate, 4 and 24 h). Baseline overall antioxidant stores were similar with all cell lines except that MnSOD^{+/+} cells had relatively lower levels (see P1 in Table 3) perhaps reflecting adaptation by the other cell lines to reduced or absent MnSOD. Incubation of MnSOD^{tet/tet} cells in doxy significantly increased antioxidant levels (see P2 in Table 3). There were no detectable differences in antioxidant levels with any of the irradiated cell lines tested immediately or 24 h. At 4 h after irradiation there was a significant decrease in total cellular antioxidant pools in each of the cell lines (see P3 in Table 3), but highest levels persisted in the MnSOD^{tet/tet} cells that were maintained in doxy.

Induction of MnSOD, but not TGF- β , Nrf2 and NF- κ B RNA Transcripts and Protein in Doxy Treated and Irradiated MnSOD^{tet/tet} Bone Marrow Stromal Cells

We next irradiated MnSOD^{tet/tet}, MnSOD^{tet/+}, MnSOD^{+/+} and MnSOD^{-/-} cell lines grown in the presence or absence of doxy to 10 Gy and harvested cells at 1 or 6 h after irradiation. RNA was extracted and analyzed by RT-PCR for mRNA as described in Materials and Methods. Protein levels were measured 6 h after irradiation. As shown in Fig. 8 and Supplementary Tables S1–S4 (<http://dx.doi.org/10.1667/RR3177.1.S1>), radiation induced MnSOD in all the cell lines except the MnSOD^{-/-} cells (see P₀ in Supplementary Table S1; <http://dx.doi.org/10.1667/RR3177.1.S1>). There was also reduced MnSOD expression in all the cell lines compared to MnSOD^{+/+} (see P_{wt} in Supplementary Table S1; <http://dx.doi.org/10.1667/RR3177.1.S1>). Growth of the MnSOD^{tet/+} and MnSOD^{tet/tet} cells in doxy resulted in increased expression of MnSOD (see P_{doxy} in Supplementary Table S1; <http://dx.doi.org/10.1667/RR3177.1.S1>). Analysis of MnSOD protein levels by Western blot demonstrated complete absence in MnSOD^{-/-} cells under any set of conditions (Fig. 9 and Supplementary Table S7; <http://dx.doi.org/10.1667/RR3177.1.S1>) and a clear induction of MnSOD in MnSOD^{tet/tet} cells by addition of doxy, irradiation alone or with doxy. Irradiation increased levels of MnSOD in all cell lines except with MnSOD^{-/-} cells.

TGF- β induction in the cell lines (with the exception of MnSOD^{-/-} cells) was similar to that with MnSOD in that radiation induced increased expression at 1 and 6 h postirradiation. With MnSOD^{tet/tet} cells, incubation in doxy for 1 h demonstrated no induction of TGF- β , and a reduction by 6 h (Fig. 8 and Supplementary Table S2; <http://dx.doi.org/10.1667/RR3177.1.S1>). In contrast, MnSOD^{+/+} as well as MnSOD^{tet/+} cells grown in doxy showed increased levels of expression of TGF- β . MnSOD^{-/-} cells had low levels of expression of TGF- β . The 6 h time point may have been early for detection of an increase in TGF protein after irradiation.

The redox sensitive promoter binding protein Nrf2 was minimally altered by radiation or doxy in all of the cell lines at 1 and 6 h (Fig. 8 and Supplementary Table S3; <http://dx.doi.org/10.1667/RR3177.1.S1>). Before irradiation, MnSOD^{+/+}, MnSOD^{tet/tet} and MnSOD^{-/-} cells had increased Nrf2 expression that were increased by radiation. Growth of MnSOD^{+/+} and MnSOD^{tet/tet} cells in the presence of doxy resulted in decreased baseline expression of Nrf2. Thus, the conditional MnSOD inducible lines MnSOD^{+/+} and MnSOD^{tet/tet} cells differed in this respect from MnSOD^{+/+} cells. MnSOD^{tet/tet} cells grown in doxy were indistinguishable from MnSOD^{+/+} cells with respect to Nrf2. Western analysis demonstrated that Nrf2 protein levels were comparable between cell lines and showed no significant effect of preincubation in doxy, irradiation or both conditions (Fig. 9).

NF- κ B RNA expression was induced by irradiation at 1 h with MnSOD^{+/+} cells. In contrast, MnSOD^{+/+}, MnSOD^{tet/tet} and MnSOD^{-/-} cells showed a significant reduction in NF- κ B expression after irradiation (Fig. 8 and Supplementary Table S4; <http://dx.doi.org/10.1667/RR3177.1.S1>). At 6 h after irradiation, NF- κ B expression was increased in MnSOD^{+/+} and MnSOD^{+/+} cells, but was still reduced in MnSOD^{tet/tet} and MnSOD^{+/+}. There was an increased baseline expression of NF- κ B in MnSOD^{tet/tet} and MnSOD^{+/+} cells, which was reduced by treating MnSOD^{tet/tet}, but not MnSOD^{-/-} cells with doxy (Fig. 8 and Supplementary Table S4; <http://dx.doi.org/10.1667/RR3177.1.S1>). Doxy treatment of MnSOD^{+/+} and MnSOD^{tet/tet} cells showed increased expression of NF- κ B after irradiation compared to untreated cells. Treatment of MnSOD^{+/+} cells with doxy decreased expression of NF- κ B. With respect to protein, there were minimal differences (Fig. 9), MnSOD^{tet/tet} cells in doxy were indistinguishable from MnSOD^{+/+} cells in NF- κ B protein. Western analysis demonstrated NF- κ B levels were decreased in all cell lines after irradiation with or without doxy pretreatment. These results may reflect the time point of 6 h, which may have been too early to detect major differences between the cell lines with respect to protein expression and may lag behind the differences detected in RNA expression (Fig. 9).

The increased expression of Nrf2 and NF- κ B in inducible lines MnSOD^{tet/+} and MnSOD^{tet/tet} as well as MnSOD^{-/-} cells compared to MnSOD^{+/+} cells may have been attributable to increased ROS levels associated with baseline decreased MnSOD. This explanation was supported by growing MnSOD^{tet/+} and MnSOD^{-/-} cells in doxy, which decreased levels of Nrf2 and NF- κ B and increased MnSOD levels. The results establish both different baseline and radiation-induced levels of RNA transcripts and proteins for stress response genes in MnSOD^{tet/tet} compared to MnSOD^{+/+} cells. The data also establish that the acute irradiation induced RNA stress response in MnSOD^{tet/tet} bone marrow stromal cell lines differed from that of both positive control MnSOD^{+/+} and negative control MnSOD^{-/-} cells.

Doxy Treatment Effects on Radiation Induced DNA Strand Breaks in MnSOD^{tet/tet} Cells

We used the neutral comet assay to measure DNA double-strand breaks in irradiated MnSOD^{tet/tet} cells. Cell lines MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} and MnSOD^{-/-} were incubated in the presence or absence of doxy for 24 h and then irradiated to 5 Gy. The cells then placed on ice 0, 10, 20 or 30 min after irradiation to terminate DNA repair. Comet tail intensity was determined at each time point and after irradiation a line was drawn connecting the 10, 20 and 30 time points. A decreased slope of the line would demonstrate faster repair of DNA strand breaks. As shown in Fig. 10 and Supplementary Table S5; <http://dx.doi.org/10.1667/RR3177.1.S1>; cells from lines MnSOD^{+/+}, MnSOD^{tet/tet} and MnSOD^{-/-} incubated in absence or presence doxy demonstrated DNA strand breaks after irradiation and were compared to MnSOD^{+/+} cells (see P₂ or P₃ in Supplementary Table S5). There was no evidence that incubation of MnSOD^{+/+} cells in doxy altered DNA repair (P₁ for slope in Supplementary Table S5), nor did doxy treated MnSOD^{tet/tet} cells show

improved repair of DNA strand breaks after irradiation (Supplementary Table S5; <http://dx.doi.org/10.1667/RR3177.1.S1>).

The results with the Comet assay (Fig. 10 and Supplementary Table S5; <http://dx.doi.org/10.1667/RR3177.1.S1>) and those measuring antioxidant stores (Table 3) establish that the MnSOD^{tet/tet} cells had adapted to a lower level of MnSOD by compensatory mechanisms; however, when MnSOD was induced by doxy treatment, upregulation of these pathways produced a response.

DISCUSSION

Intracellular levels of mitochondrial targeted MnSOD have been shown to greatly influence cellular, tissue and organ radiosensitivity (1, 2, 6, 23, 24) and represent a first line of defense against ionizing radiation induced ROS (2, 6). Intrinsically low levels of MnSOD can be compensated by upregulation of other intracellular antioxidants including acute elevation of glutathione and other small molecule free radical scavengers or upregulation of other ROS neutralizing enzymes (1, 2, 6). Conversely, overexpression of MnSOD in transgenic mice leads to down-modulation of other antioxidant defenses, with no intrinsic radioresistance (26). In contrast, acute transgene-mediated overexpression of MnSOD confers tissue specific or total body radioresistance (25–28).

To elucidate the molecular biologic changes that follows acute upregulation of MnSOD, we designed a tet-inducible mouse strain in which transcription of MnSOD and induction of protein is regulated by the tet-promoter (35). In the present report, we describe the radiobiological characteristics of reversible induction of MnSOD in tet-inducible mouse bone marrow stromal cell lines. Incubation of MnSOD^{tet/tet} cells in doxy induced a significant elevation of MnSOD RNA within 4 h while removal of doxy caused a detectable decrease by 4 h.

MnSOD^{tet/tet} bone marrow stromal cells demonstrated intrinsically low levels of MnSOD, compared to the complete absence in genetically null embryo fibroblast cells (21, 22, 27). However, the low levels of MnSOD were not sufficient to normalize radioresistance in clonogenic survival curve assay, nor cell killing in plateau phase culture assay compared to that in heterozygous MnSOD^{tet/+} or wild-type MnSOD^{+/+} cell lines. There was a clear increase in the radiation resistance of MnSOD^{tet/tet} cells when MnSOD levels were elevated by growth in doxy. In contrast to other MnSOD-level-dependent radiobiologic parameters in MnSOD^{tet/tet} cell lines, radiation-induced cell cycle arrest was unrelated to MnSOD levels. Total intracellular antioxidant levels in all cell lines with exception of MnSOD^{+/+} cells showed uniform irradiation induced decrease.

There were intrinsically low levels of antioxidants in MnSOD^{+/+} wild-type cells compared to MnSOD^{-/-} or MnSOD^{tet/tet} cells not in doxy. These data are consistent with the notion that baseline levels of MnSOD represent a first line of defense against ROS and allow intracellular levels of glutathione and other small molecule antioxidants to be reduced. Maintaining a normal cellular antioxidant balance has been one strategy for small molecule radioprotective drug discovery (38–41). In wild-type cells, MnSOD RNA (Fig. 8) but not protein (Fig. 9) increased after irradiation, perhaps because of the possible disconnect in time between increased transcription and translation to reveal higher protein levels, which is sometimes seen in wild-type cells.

The differences in initial DNA double-strand break damage at 0 and 10 min after exposure to 5 Gy were different between cell lines. The removal of damage assessed by mean comet lengths from 10–30 min after irradiation also changed little between the cell lines, suggesting that initial double-strand break repair damage correlated better with the

sensitivity of MnSOD manipulated cells rather than with DNA repair. Therefore, we cannot conclude that there was an improvement in DNA repair in MnSOD manipulated cells.

In the present Tet-On cellular model, elevation of MnSOD level of expression was dependent upon doxy for cells in tissue culture. While doxy is itself a mild radioprotector (44, 45), this model system appears to be more easily controllable than that in a previous publication (46) in which a Tet-Off system was reported. In a previous publication, heterozygous mice maintained on doxy had a decrease in both MnSOD RNA transcription and protein; however, the system was not tightly controlled. The present MnSOD^{tet/tet} mouse derived cell lines present a system suitable for conditional upregulation and bioavailability of the MnSOD gene product *in vitro*. The availability of doxy inducible mouse bone marrow stromal cell lines may also allow investigation of the role of MnSOD levels in the differentiation of bone marrow stromal cells to osteoblasts, chondrocytes, adipocytes and other lineages associated with the mesenchymal stem cell phenotype (36).

Preliminary studies show that implants of slow-release doxy pellets can keep MnSOD^{tet/tet} newborn mice alive through weaning and that adult MnSOD^{tet/tet} mice can be maintained with doxy in drinking water. Studies with MnSOD^{tet/tet} mice should allow determination of the role of MnSOD levels in responses to ionizing radiation including stem cell self-renewal, carcinogenesis and organ specific toxicity. The present MnSOD^{tet/tet} cell lines should be valuable for study of the role of MnSOD in the molecular mechanisms involved in mammalian cellular radiobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH Grants CA-R01-CA119927 and NIAID U19-A1068021.

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FIG. 1.

Generation and genotyping of *MnSOD^{tet}* allele. Panel A: Schematic of mutagenesis approach to generate tetracycline-regulated *MnSOD* allele. The top line is endogenous *MnSOD* allele, comprised of five exons (filled rectangles). The middle line is linearized targeting plasmid with Tet-On regulatory cassette inserted in exon 1 approximately 30 nucleotides 5' of initiation codon. rtTA is coding sequence of reverse tetracycline repressor protein, neo^R is G418 selectable marker gene, and tetO+CMV is comprised of five copies of tetracycline operator 5' of minimal CMV promoter. Homologous recombination between *MnSOD* allele and targeting plasmid in ES cells resulted in mutant *MnSOD^{tet}* allele (bottom line). Panel B: Southern blot of *Eco*RI-digested DNA from tails of offspring of cross between heterozygous *MnSOD^{tet/+}* parents. Blot was hybridized with Probe A located 5' of the *MnSOD* locus and not present in the targeting plasmid. Panel C: PCR genotyping assay to distinguish wild-type *MnSOD* and *MnSOD^{tet}* alleles. 473-bp PCR product from MnSODwtF and MnSODwtR primers corresponds to wild-type *MnSOD* allele. 281-bp PCR product from MnSODwtF and MnSODTetR primers corresponds to *MnSOD^{tet}* allele.

FIG. 2.

Expression of MnSOD protein in MnSOD^{tet/tet} cell lines. Western analysis (as described in Materials and Methods) was performed on cell lines from MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} demonstrating reduced MnSOD expression in MnSOD^{tet/+} (1.8 ± 0.1 -fold, $P = 0.0125$) and MnSOD^{tet/tet} (55.1 ± 2.5 -fold, $P = 0.0022$) relative to MnSOD^{+/+} cells. Cells from a negative control, MnSOD^{-/-} cells had no detectable reactive protein.

A. Induction of MnSOD mRNA by Doxycycline in MnSOD^{tet/tet} bone marrow stromal cells

B. Reduced MnSOD gene expression after Doxy removal from MnSOD^{tet/tet} cells

FIG. 3.

Time dependence of doxy mediated induction and loss of expression of MnSOD RNA in MnSOD^{tet/tet} cells. Cells were incubated in 0.25 $\mu\text{g/ml}$ doxy for times ranging from 0 to 48 h then harvested, RNA extracted and RT-PCR performed for MnSOD mRNA expression. Panel A: Increased RNA detected 4–12 h after addition of doxy. Panel B: Removal of doxy after 24 h in doxy decreases MnSOD mRNA within 1 h and significantly by 4 h.

FIG. 4.

Doxy induction of MnSOD biochemical activity in MnSOD^{tet/tet} bone marrow stromal cells. Units of biochemical activity are shown. In no doxy, both MnSOD^{tet/+} and MnSOD^{tet/tet} cells had lower MnSOD levels than MnSOD^{+/+} cells (* $P=0.0297$ and $P=0.0108$, respectively). When grown in doxy, MnSOD^{tet/+} and MnSOD^{tet/tet} cells showed elevated MnSOD levels compared to the same cells in no doxy (# $P=0.0405$ and $P=0.0135$, respectively). Furthermore, in doxy both MnSOD^{tet/+} and MnSOD^{tet/tet} cells achieved levels of biochemically active MnSOD comparable to the levels in MnSOD^{+/+} control cells ($P=0.3180$ and $P=0.1602$, respectively).

FIG. 5.

Immunofluorescence detection of MnSOD in MnSOD^{tet/tet} cells grown in the presence or absence of doxy. Immunolocalization of MnSOD in cell lines was carried out on coverslip preparations as described in the Materials and Methods. Each image pair shows MnSOD staining (green) on the left and MnSOD with actin (red) and nuclear staining DAPI (blue) on the right. MnSOD^{tet/tet} cells incubated in absence of doxy (panels A and B) or presence of doxy (0.25 μg/ml) doxy (panels C and D). MnSOD^{+/+} cells incubated in absence of doxy (panels E and F) and presence of 0.25 μg/ml doxycycline (panels G and H). 60× magnification.

FIG. 6. Effect of doxy on clonogenic radiation survival curves of MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{+/+} mouse bone marrow stromal cell lines. MnSOD^{+/+} (panel A), MnSOD^{tet/+} and MnSOD^{tet/tet} (panel B) cells were grown in the presence or absence of 0.25 μ g/ml doxy for 24 h prior to irradiation and plating. Doxy was added to the doxy cultures every 48 h until the plates were stained on day 8. The D₀ (panel C) and $\tilde{n}$ (panel D) for the curves are shown. MnSOD^{tet/tet} cells were radiosensitive in no doxy compared to MnSOD^{+/+} cells (**P* = 0.0175). MnSOD^{tet/tet} cells in doxy became radioresistant relative to the same cells in no doxy (*P* = 0.0060). MnSOD^{tet/tet} cells were comparable in radioresistance to MnSOD^{+/+} cells when both were in doxy (*P* = 0.4414).

FIG. 7.

MnSOD^{+/+} and MnSOD^{tet/tet} cells demonstrate G₂/M-phase arrest after irradiation. MnSOD^{+/+} (panel A) and MnSOD^{tet/tet} (panel B) cells grown in the presence or absence of doxy for 24 h were irradiated to 5 or 10 Gy. Cells were harvested 24 h later and cell cycle analysis was performed. A G₂/M-phase arrest was detected in both cells grown in the presence or absence of doxy.

FIG. 8.

Radiation induction of mRNA for MnSOD, TGF- β , Nrf2 and NF- κ B in MnSOD^{tet/tet} cells in the presence or absence of doxy. Harvested and cytocentrifuged cell packs from MnSOD^{+/+}, MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{-/-} were assayed at 1 and 6 h after 10 Gy irradiation by RT-PCR as described in the Materials and Methods. Results are shown for panel A: MnSOD, panel B: TGF- β , panel C: Nrf2 and panel D: NF- κ B. (Statistical analysis is shown in Supplementary Tables S1–S4: <http://dx.doi.org/10.1667/RR3177.1.S1>)

FIG. 9.

Protein levels of MnSOD, TGF- β , Nrf2 and NF- κ B in control and irradiated MnSOD^{tet/tet} cell lines. MnSOD^{+/+}, MnSOD^{tet/+}, MnSOD^{tet/tet} and MnSOD^{-/-} cell lines were incubated in the presence or absence of doxy for 24 h, then irradiated to 0 or 10 Gy. Six hours later cells were harvested and proteins were subjected to Western analysis for MnSOD, TGF- β , Nrf2 and NF- κ B. Expression of GAPDH was used for a loading control. (Densitometry is shown in Supplementary Table S7: <http://dx.doi.org/10.1667/RR3177.1.S1>)

FIG. 10.

Improved DNA repair by comet assay on doxy treated MnSOD^{tet/tet} bone marrow stromal cell lines. Cells from MnSOD^{tet/tet}, MnSOD^{tet/+}, MnSOD^{+/+} and MnSOD^{-/-} lines were irradiated to 5 Gy and analyzed for DNA damage immediately using a neutral Comet Tail Length Assay as described in the Materials and Methods. (Statistical analysis is shown in Supplementary Table S5: <http://dx.doi.org/10.1667/RR3177.1.S1>)

Effect of MnSOD Gene Expression on Cell Viability and Total Number of Plateau Phase MnSOD^{tet/tet} Bone Marrow Stromal Cells at 7 Days after Irradiation

TABLE 1

Cell line	Dose (Gy)	Viability (mean $\pm$ SD, n is the sample size)		Total number of cells ($\times 10^6$) (mean $\pm$ SD, n is the sample size)	
		without doxycycline	with doxycycline	without doxycycline	with doxycycline
MnSOD ^{+/+}	0	95.8 $\pm$ 2.0 (n = 3)	94.8 $\pm$ 1.1 (n = 3)	7.9 $\pm$ 2.1 (n = 3)	1.7 $\pm$ 0.1 (n = 3)
	2	94.3 $\pm$ 4.4 (n = 3)	93.8 $\pm$ 1.6 (n = 3)	8.4 $\pm$ 2.6 (n = 3)	1.4 $\pm$ 0.2 (n = 3)
	4	94.2 $\pm$ 4.9 (n = 3)	94.3 $\pm$ 1.8 (n = 3)	8.7 $\pm$ 3.6 (n = 3)	1.3 $\pm$ 0.1 (n = 3)
	6	93.6 $\pm$ 5.6 (n = 3)	94.1 $\pm$ 0.9 (n = 3)	8.3 $\pm$ 2.8 (n = 3)	1.2 $\pm$ 0.1 (n = 3)
	8	93.5 $\pm$ 6.0 (n = 3)	93.4 $\pm$ 0.8 (n = 3)	7.4 $\pm$ 2.2 (n = 3)	1.1 $\pm$ 0.2 (n = 3)
	10	92.2 $\pm$ 6.7 (n = 3)	92.7 $\pm$ 2.2 (n = 3)	7.9 $\pm$ 3.8 (n = 3)	1.1 $\pm$ 0.2 (n = 3)
	Slope *	-0.30 (0.33)	-0.17 (0.33)	-0.05 (0.06)	-0.06 (0.06)
MnSOD ^{tet/tet}	P value	P3 = 0.7766		P3 = 0.9025	
	0	95.0 $\pm$ 2.8 (n = 3)	96.4 $\pm$ 0.5 (n = 3)	5.1 $\pm$ 1.2 (n = 3)	2.4 $\pm$ 0.3 (n = 3)
	2	88.4 $\pm$ 12.6 (n = 3)	96.8 $\pm$ 0.4 (n = 3)	5.1 $\pm$ 1.7 (n = 3)	2.4 $\pm$ 0.2 (n = 3)
	4	87.3 $\pm$ 10.6 (n = 3)	96.8 $\pm$ 1.0 (n = 3)	4.1 $\pm$ 1.5 (n = 3)	2.3 $\pm$ 0.1 (n = 3)
	6	85.2 $\pm$ 15.1 (n = 3)	97.1 $\pm$ 0.9 (n = 3)	3.5 $\pm$ 0.6 (n = 3)	1.9 $\pm$ 0.2 (n = 3)
	8	84.6 $\pm$ 12.3 (n = 3)	96.0 $\pm$ 1.1 (n = 3)	3.8 $\pm$ 2.2 (n = 3)	1.8 $\pm$ 0.1 (n = 3)
	10	84.4 $\pm$ 13.0 (n = 3)	96.5 $\pm$ 0.4 (n = 3)	3.0 $\pm$ 1.0 (n = 3)	1.7 $\pm$ 0.1 (n = 3)
	Slope *	-0.94 (0.33)	-0.02 (0.33)	-0.21 (0.06)	-0.08 (0.06)
MnSOD ^{+/+}	P value	P1 = 0.1673, P2 = 0.7437, P3 = 0.0475		P1 = 0.0476, P2 = 0.7956, P3 = 0.1082	
	0	94.2 $\pm$ 2.7 (n = 3)	85.1 $\pm$ 1.4 (n = 3)	2.4 $\pm$ 1.9 (n = 3)	4.1 $\pm$ 0.6 (n = 3)
	2	89.9 $\pm$ 11.1 (n = 3)	86.1 $\pm$ 1.3 (n = 3)	1.5 $\pm$ 0.8 (n = 3)	4.1 $\pm$ 0.6 (n = 3)
	4	81.1 $\pm$ 26.5 (n = 3)	86.4 $\pm$ 0.8 (n = 3)	1.2 $\pm$ 0.4 (n = 3)	4.0 $\pm$ 0.3 (n = 3)
	6	81.7 $\pm$ 25.1 (n = 3)	86.6 $\pm$ 0.3 (n = 3)	1.1 $\pm$ 0.3 (n = 3)	3.5 $\pm$ 0.2 (n = 3)
	8	80.6 $\pm$ 23.0 (n = 3)	85.8 $\pm$ 2.5 (n = 3)	1.1 $\pm$ 0.4 (n = 3)	3.6 $\pm$ 0.6 (n = 3)
	10	83.2 $\pm$ 20.1 (n = 3)	85.6 $\pm$ 1.7 (n = 3)	1.2 $\pm$ 0.6 (n = 3)	3.4 $\pm$ 0.2 (n = 3)
	Slope *	-1.18 (0.33)	0.02 (0.28)	-0.10 (0.06)	-0.08 (0.06)
MnSOD ^{-/-}	P value	P1 = 0.0608, P2 = 0.6741, P3 = 0.0105		P1 = 0.5070, P2 = 0.7902, P3 = 0.7830	
	0	94.1 $\pm$ 3.7 (n = 3)	96.0 $\pm$ 0.5 (n = 3)	3.4 $\pm$ 0.8 (n = 3)	4.5 $\pm$ 0.8 (n = 3)
	2	93.7 $\pm$ 4.1 (n = 3)	95.7 $\pm$ 1.2 (n = 3)	3.2 $\pm$ 0.3 (n = 3)	4.4 $\pm$ 1.0 (n = 3)

Cell line	Dose (Gy)	Viability (mean $\pm$ SD, n is the sample size)		Total number of cells ($\times 10^6$) (mean $\pm$ SD, n is the sample size)	
		without doxycycline	with doxycycline	without doxycycline	with doxycycline
	4	92.7 $\pm$ 4.4 (n = 3)	96.2 $\pm$ 0.6 (n = 3)	2.8 $\pm$ 0.6 (n = 3)	4.6 $\pm$ 0.8 (n = 3)
	6	87.8 $\pm$ 13.3 (n = 3)	95.5 $\pm$ 0.9 (n = 3)	2.7 $\pm$ 0.2 (n = 3)	4.2 $\pm$ 0.9 (n = 3)
	8	87.7 $\pm$ 12.6 (n = 3)	94.9 $\pm$ 1.2 (n = 3)	2.4 $\pm$ 0.2 (n = 3)	4.2 $\pm$ 0.8 (n = 3)
	10	88.8 $\pm$ 10.8 (n = 3)	93.3 $\pm$ 2.6 (n = 3)	2.5 $\pm$ 0.5 (n = 3)	3.3 $\pm$ 1.7 (n = 3)
Slope*		-0.70 (0.33)	-0.23 (0.33)	-0.10 (0.06)	-0.10 (0.06)
P value		P1 = 0.3861, P2 = 0.8936, P3 = 0.3096		P1 = 0.5508, P2 = 0.6291, P3 = 0.9930	

Notes. Cells from all of the cell lines were grown to confluence and incubated for 24 h in 0 or 0.25 μ g/ml doxycycline for 24 h at which time the cells were irradiated to doses ranging from 0 to 10 Gy. At day 7 or 14 (Table 2) after irradiation, the viability and total number of cells was determined. The viability is indicated by mean $\pm$ standard deviation (SD) for each subgroup, which is shown for each cell line, doxycycline treatment, radiation dose and day of measurement. At day 7 or 14, a linear mixed model was built on each endpoint, using group and dose and their interaction term as fixed explanatory variables, and the experiment ID was used as a random effect (triplicate experiments were performed). Each group is defined by the combination of cell line (MnSOD^{+/+}, MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{-/-} cells) and treatment (with or without doxycycline). The F test was used to compare slopes between any two groups. A significant *P* value of less than 0.05 indicates a significant difference in the dose effect between any two groups. *P* values were not adjusted for multiple comparisons. Total cell numbers were analyzed in the same way. Analyses were performed using SAS software. P1 is the *P* value comparing each cell line incubated in the absence of doxycycline with MnSOD^{+/+} cells, P2 is the *P* value comparing each cell line incubated in the presence of doxycycline to MnSOD^{-/-}, P3 is the *P* value for each cell line comparing growth in absence of doxycycline to the same cells grown in the presence of doxycycline. Significant differences are shown in *italicized* (42, 43).

* Slope is the increase in viability or total number of cells ($\times 10^6$) for every 1 Gy increase in radiation dose.

Effect of MnSOD Gene Expression on Cell Viability and Total Cell Number of Plateau Phase MnSOD^{tet/tet} Bone Marrow Stromal Cells at 14 Days after Irradiation

TABLE 2

Cell line	Dose (Gy)	Viability (mean $\pm$ SD, n is the sample size)		Total number of cells ($\times 10^6$) (mean $\pm$ SD, n is the sample size)	
		without doxycycline	with doxycycline	without doxycycline	with doxycycline
MnSOD ^{-/-}	0	93.1 $\pm$ 2.9 (n = 3)	94.3 $\pm$ 0.9 (n = 3)	6.7 $\pm$ 4.7 (n = 3)	1.5 $\pm$ 0.2 (n = 3)
	2	92.7 $\pm$ 3.7 (n = 3)	93.0 $\pm$ 1.8 (n = 3)	6.7 $\pm$ 3.3 (n = 3)	1.3 $\pm$ 0.1 (n = 3)
	4	92.3 $\pm$ 5.0 (n = 3)	94.9 $\pm$ 1.5 (n = 3)	6.1 $\pm$ 2.6 (n = 3)	1.3 $\pm$ 0.1 (n = 3)
	6	91.1 $\pm$ 3.3 (n = 3)	93.6 $\pm$ 2.0 (n = 3)	5.7 $\pm$ 1.6 (n = 3)	1.1 $\pm$ 0.0 (n = 3)
	8	90.6 $\pm$ 6.5 (n = 3)	92.1 $\pm$ 3.2 (n = 3)	5.2 $\pm$ 1.8 (n = 3)	0.9 $\pm$ 0.1 (n = 3)
	10	91.1 $\pm$ 4.9 (n = 3)	94.3 $\pm$ 2.9 (n = 3)	5.0 $\pm$ 2.0 (n = 3)	0.9 $\pm$ 0.0 (n = 3)
	Slope *	-0.25 (0.24)	-0.06 (0.24)	-0.19 (0.09)	-0.06 (0.09)
	P value	P3 = 0.5727		P3 = 0.3008	
	0	93.1 $\pm$ 4.3 (n = 3)	90.0 $\pm$ 3.4 (n = 3)	5.5 $\pm$ 2.2 (n = 3)	3.0 $\pm$ 0.9 (n = 3)
	2	93.3 $\pm$ 2.9 (n = 3)	90.4 $\pm$ 1.6 (n = 3)	7.9 $\pm$ 4.7 (n = 3)	2.6 $\pm$ 0.7 (n = 3)
MnSOD ^{tet/tet}	4	91.1 $\pm$ 4.8 (n = 3)	93.0 $\pm$ 1.2 (n = 3)	4.5 $\pm$ 1.1 (n = 3)	2.3 $\pm$ 0.8 (n = 3)
	6	91.8 $\pm$ 5.7 (n = 3)	91.4 $\pm$ 2.1 (n = 3)	4.3 $\pm$ 1.2 (n = 3)	2.1 $\pm$ 0.5 (n = 3)
	8	85.2 $\pm$ 8.6 (n = 3)	91.9 $\pm$ 0.8 (n = 3)	3.6 $\pm$ 0.8 (n = 3)	1.6 $\pm$ 0.4 (n = 3)
	10	78.7 $\pm$ 17.4 (n = 3)	92.3 $\pm$ 1.7 (n = 3)	2.2 $\pm$ 0.9 (n = 3)	1.6 $\pm$ 0.2 (n = 3)
	Slope *	-1.36 (0.24)	0.20 (0.24)	-0.42 (0.09)	-0.15 (0.09)
	P value	P1 = 0.0011, P2 = 0.4312, P3 < 0.0001		P1 < 0.0610, P2 = 0.4944, P3 = 0.0266	
	0	91.4 $\pm$ 4.8 (n = 3)	83.5 $\pm$ 2.1 (n = 3)	3.2 $\pm$ 2.0 (n = 3)	3.1 $\pm$ 0.5 (n = 3)
	2	88.8 $\pm$ 3.8 (n = 3)	85.5 $\pm$ 3.9 (n = 3)	3.3 $\pm$ 2.7 (n = 3)	3.6 $\pm$ 0.7 (n = 3)
	4	88.0 $\pm$ 5.6 (n = 3)	85.0 $\pm$ 3.5 (n = 3)	2.7 $\pm$ 2.2 (n = 3)	3.4 $\pm$ 0.4 (n = 3)
	6	88.0 $\pm$ 7.6 (n = 3)	85.0 $\pm$ 1.9 (n = 3)	2.7 $\pm$ 2.6 (n = 3)	3.0 $\pm$ 0.5 (n = 3)
MnSOD ^{+/+}	8	85.7 $\pm$ 8.7 (n = 3)	85.1 $\pm$ 3.8 (n = 3)	2.8 $\pm$ 2.7 (n = 3)	3.2 $\pm$ 0.7 (n = 3)
	10	88.0 $\pm$ 8.2 (n = 3)	85.3 $\pm$ 1.7 (n = 3)	2.4 $\pm$ 2.0 (n = 3)	3.3 $\pm$ 0.5 (n = 3)
	Slope *	-0.38 (0.24)	0.12 (0.24)	-0.08 (0.09)	-0.01 (0.09)
	P value	P1 = 0.7036, P2 = 0.5972, P3 = 0.1423		P1 = 0.3747, P2 = 0.6511, P3 = 0.5488	
	0	88.8 $\pm$ 6.0 (n = 3)	93.3 $\pm$ 3.0 (n = 3)	4.2 $\pm$ 1.2 (n = 3)	5.6 $\pm$ 2.2 (n = 3)
	2	88.8 $\pm$ 5.7 (n = 3)	91.7 $\pm$ 5.2 (n = 3)	4.0 $\pm$ 1.8 (n = 3)	5.1 $\pm$ 1.9 (n = 3)

Cell line	Dose (Gy)	Viability (mean $\pm$ SD, n is the sample size)		Total number of cells ($\times 10^6$) (mean $\pm$ SD, n is the sample size)	
		without doxycycline	with doxycycline	without doxycycline	with doxycycline
4		87.6 $\pm$ 9.5 (n = 3)	93.0 $\pm$ 2.4 (n = 3)	3.1 $\pm$ 0.5 (n = 3)	5.0 $\pm$ 1.3 (n = 3)
6		87.7 $\pm$ 8.3 (n = 3)	93.2 $\pm$ 1.9 (n = 3)	2.9 $\pm$ 0.7 (n = 3)	4.5 $\pm$ 1.4 (n = 3)
8		86.2 $\pm$ 9.7 (n = 3)	90.9 $\pm$ 2.8 (n = 3)	2.8 $\pm$ 0.6 (n = 3)	4.1 $\pm$ 1.3 (n = 3)
10		84.8 $\pm$ 7.9 (n = 3)	91.6 $\pm$ 1.4 (n = 3)	2.4 $\pm$ 0.5 (n = 3)	4.0 $\pm$ 1.1 (n = 3)
Slope*		-0.23 (0.24)	-0.11 (0.24)	-0.18 (0.09)	-0.16 (0.09)
P value		P1 = 0.9648, P2 = 0.8721, P3 = 0.7195		P1 = 0.9294, P2 = 0.4093, P3 = 0.9029	

Note. P values are explained in the notes of Table 1.

* Slope is the increase in viability or total number of cells ($\times 10^6$) for every 1 Gy increase in dose.

TABLE 3

Antioxidant Status (Total Antioxidant Capacity) 4 h and 24 h after 10 Gy Irradiation of MnSOD^{tet/tet} Cells

Cell line	Trolox units per mg protein		
	0 Gy	4 h after 10 Gy	24 h after 10 Gy
MnSOD ^{-/-}	0.106 ± 0.001	0.046 ± 0.001	<i>0.159 ± 0.013</i>
		<i>P3 = 0.0003</i>	<i>P3 = 0.1196</i>
MnSOD ^{-/-} + doxy	0.119 ± 0.008	0.045 ± 0.001	<i>0.222 ± 0.042</i>
	<i>P2 = 0.2403</i>	<i>P3 = 0.0147</i>	<i>P3 = 0.1075</i>
MnSOD ^{tet/+}	0.181 ± 0.015	0.096 ± 0.001	<i>0.175 ± 0.083</i>
	<i>P1 = 0.0379</i>	<i>P3 = 0.0299</i>	<i>P3 = 0.9532</i>
MnSOD ^{tet/+} + doxy	0.151 ± 0.015	0.083 ± 0.007	<i>0.243 ± 0.093</i>
	<i>P2 = 0.2929</i>	<i>P3 = 0.0450</i>	<i>P3 = 0.6168</i>
MnSOD ^{tet/tet}	0.158 ± 0.002	0.087 ± 0.013	<i>0.199 ± 0.044</i>
	<i>P1 = 0.0018</i>	<i>P3 = 0.0308</i>	<i>P3 = 0.5250</i>
MnSOD ^{tet/tet} + doxy	0.259 ± 0.033	0.123 ± 0.003	<i>0.406 ± 0.049</i>
	<i>P2 = 0.0276</i>	<i>P3 = 0.0125</i>	<i>P3 = 0.2929</i>
MnSOD ^{-/-}	0.130 ± 0.001	0.047 ± 0.002	<i>0.159 ± 0.090</i>
	<i>P1 = 0.0035</i>	<i>P3 = 0.0011</i>	<i>P3 = 0.1940</i>
MnSOD ^{-/-} + doxy	0.126 ± 0.005	0.071 ± 0.001	<i>0.141 ± 0.063</i>
	<i>P2 = 0.5149</i>	<i>P3 = 0.0087</i>	<i>P3 = 0.2346</i>

Notes. Cells from the MnSOD^{+/+}, MnSOD^{tet/tet}, MnSOD^{tet/+} and MnSOD^{-/-} cell lines were grown in the presence or absence of 0.25 µg/ml doxycycline for 24 h and then were irradiated to 10 Gy. At three times (immediately after irradiation, 4 h and 24 h later), cells were harvested for assay of total antioxidant capacity. Antioxidant levels are expressed as trolox units/mg protein. Results are shown for the 4 and 24 h times. P1 is the *P* value comparing either MnSOD^{tet/+}, MnSOD^{tet/tet} or MnSOD^{-/-} cells at 0 Gy to the MnSOD^{+/+} cell lines. P2 is the *P* value which compares each cell line to itself grown in the presence or absence of doxycycline. P3 compares each cell line grown under the same conditions (doxy or none) at 10 Gy with 0 Gy. Significant *P* values are *italicized*.