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Radioprotection *In Vitro* and *In Vivo* by Minicircle Plasmid Carrying the Human Manganese Superoxide Dismutase Transgene

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Abstract

Manganese superoxide dismutase plasmid liposomes (MnSOD-PL) confer organ-specific *in vivo* ionizing irradiation protection. To prepare for potential intravenous clinical trials of systemic MnSOD-PL for radioprotection in humans, plasmid and bacterial sequences were removed and a new minicircle construct was tested. Minicircle MnSOD was purified and then cotransfected into 32D cl 3 murine interleukin-3-dependent hematopoietic progenitor cells along with another plasmid carrying the *neo* gene. Cells were selected in G418 (50 μg/ml) and cloned by limiting dilution. Biochemical analysis of minicircle MnSOD-transfected cells showed an MnSOD biochemical activity level of 5.8 ± 0.5 U/mg compared with 2.7 ± 0.1 U/mg for control 32D cl 3 cells (p = 0.0039). 32D-mc-MnSOD cells were as radioresistant as full-length MnSOD-PL transgene-expressing 2C6 cells, relative to 32D cl 3 parent cells, with an increased shoulder on the radiation survival curve ($\overline{n}$ = 4.8 ± 0.2 and $\overline{n}$ = 4.6 ± 0.2, respectively, compared with 1.5 ± 0.5 for 32D cl 3 cells; p = 0.007). C57BL/6NHsd mice received intraoral mc-MnSOD-PL, mc-DsRed-PL control, full-length MnSOD-PL, or blank-PL and then were irradiated 24 hr later with 31 Gy to the esophagus. Mice receiving mc-MnSOD-PL showed increased survival compared with control mice or mice treated with mc-DsRed-PL (p = 0.0003 and 0.039, respectively), and comparable to full-length MnSOD-PL. Intravenous, systemic administration of mc-MnSOD-PL protected mice from total body irradiation (9.75 Gy). Therefore, minicircle DNA containing the human MnSOD transgene confers undiminished radioprotection *in vitro* and *in vivo*.

Introduction

Ionizing irradiation induces cellular, tissue, and organ toxicity by mechanisms that include induction of radical oxygen species (ROS) (Hahn *et al*., 2000; Weiss and Landauer, 2000; Mitchell and Krishna, 2002; Epperly *et al*., 2004; Spitz *et al*., 2004). Antioxidant defenses include the bioavailability of ROS-neutralizing enzymes, which modulate ionizing irradiation-induced damage (Spitz *et al*., 2004). In particular, three forms of superoxide dismutase, two extracellular copper/zinc metalloenzymes, and one mitochondria-targeted manganese metalloenzyme, dismutate superoxide to hydrogen peroxide, which is then further acted on by enzymes including catalase and glutathione peroxidase to form water (Epperly *et al*., 2004). One approach toward increasing cellular and tissue defenses against ionizing irradiation has been through use of a gene therapy technique to overexpress mitochondrial manganese superoxide dismutase before irradiation exposure or intermittently during fractionated irradiation (Epperly *et al*., 2001a, 2001b, 2003c; Greenberger *et al*., 2003; Greenberger and Epperly, 2004, 2007; Carpenter *et al*., 2005).

For therapeutic application of a gene therapy technique in radiation protection, transgene expression during the time of irradiation exposure is desirable, and should be followed by clearing of the transgene cDNA, as well as RNA and protein, from the system (Epperly *et al*., 2000, 2003c; Greenberger *et al*., 2003; Greenberger and Epperly, 2004, 2007). Plasmid liposomes have offered an attractive vehicle by which to achieve such transient overexpression, and this technique of antioxidant gene therapy has been shown to be effective *in vitro* and *in vivo* for organ-specific radioprotection of the oral cavity (Guo *et al*., 2003; Epperly *et al*., 2007a), esophagus (Epperly *et al*., 2001a, 2001b), lung (Carpenter *et al*., 2005), and bladder (Kanai *et al*., 2002).

Systemic, intravenous administration of plasmid liposomes has been reported to be associated with an inflam-

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matory response that may be attributable to CpG sequences within the bacterial plasmid backbone of the construct (Sellens *et al*., 2005). A novel approach has been used to create minicircle plasmid constructs in a manner that eliminates the bacterial CpG sequences (Chen *et al*., 2005). In the present studies, we sought to determine whether minicircle plasmid liposomes containing the human manganese superoxide dismutase (MnSOD) transgene could be used for *in vitro* and *in vivo* radioprotection and whether this construct eliminated the inflammatory response through removal of bacterial CpG sequences while retaining therapeutic transfer and transient expression of MnSOD biochemical activity.

Materials and Methods

Construction of minicircle MnSOD plasmid

The minicircle MnSOD vector is based on p2ϕC31, the minicircle parent plasmid DNA vector, constructed by Z.-Y. Chen (Chen *et al*., 2005). The human MnSOD transgene was amplified by polymerase chain reaction (PCR) from plasmid pNGVL3-MnSOD. The 1482-bp human MnSOD gene expression cassette, including a human cytomegalovirus (CMV) promoter, human MnSOD gene, and a poly(A) tail, was inserted into the site between *Spe*I and *Xho*I excision points in the p2ϕC31 plasmid, resulting in plasmid p2ϕC31.MnSOD (Fig. 1). A control minicircle construct containing the DsRed (Discosoma sp. red fluorescent protein) transgene was prepared according to the same procedures.

Minicircle production

The protocol for minicircle plasmid isolation as previously described by Chen and coworkers (2005) was used to produce minicircle MnSOD. Plasmid p2ϕC31.MnSOD or p2ϕC31.DsRed was transformed into *Escherichia coli* TOP 10 (Chen *et al*., 2005). A single colony was grown overnight in fresh Luria–Bertani (LB) broth at 37°C and then pelleted in a centrifuge (Avanti J-20 XP; Beckman Coulter, Fullerton, CA) at 3000 rpm for 20 min at 20°C. The pellet was resuspended at 4:1 (v/v) in fresh LB broth containing 1% L-arabinose. The bacteria were incubated at 32°C with shaking at 250 rpm for 2 hr, at which time an additional 1.5 volumes of fresh LB broth (pH 8.0, containing 1% L-arabinose) was added and the incubation was continued at the increased temperature of 37°C for 2 hr. Minicircle DNA was prepared according to the instructions included with the plasmid purification kits from Qiagen (Valencia, CA). Agarose gel electrophoresis was used for minicircle DNA purification to obtain 100% pure minicircle DNA.

Minicircle MnSOD DNA sequencing

Minicircle MnSOD DNA (p2ϕC31.MnSOD) was sequenced with an ABI PRISM 3700 DNA sequencer (Applied Biosystems, Foster City, CA) to confirm that the minicircles contained the MnSOD expression cassette.

Cell lines and cell culture

The 32D cl 3 mouse hematopoietic progenitor cell line, dependent for growth on interleukin (IL)-3, has been described previously (Epperly *et al*., 2002, 2003b). 32D cl 3 cells were passaged in fresh RMPI 1640 medium containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin–streptomycin, and 15% WEHI-3 conditioned medium as a source of IL-3. Twenty-four hours later, 1×10^7 32D cl 3 cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 0.25 ml of PBS. For each electroporation, 5 μg (1 μg/μl) of p2ϕC31.MnSOD or p2ϕC31.DsRed and 0.5 μg of pSV2-neo were added to the 32D cl 3 cells and transferred to an electroporation cuvette (cat. no. 165-2088; Bio-Rad). A Bio-Rad Gene Pulser II set to 280 V and 950 μF was used to electroporate the cells. The cells were then placed on ice for 20 min and transferred to a flask containing prewarmed medium. Two days after electroporation, transfected cells were selected by addition of G418 (500 mg/μl) according to published methods (Epperly *et al*., 2002). A clonal line was selected by limiting dilution and expanded as previously described (Epperly *et al*., 2002). Subclones expressing the MnSOD or DsRed transgenes were identified by RT-PCR, using primers specific for MnSOD or DsRed as previously described (Epperly *et al*., 2002). A subclone of 32D cl 3, overexpressing the human MnSOD transgene (2C6), has been reported previously (Epperly *et al*., 2002, 2003b). Both cell lines were grown in WEHI-3 cell-conditioned medium as a source of IL-3, in a high-humidity incubator, in RMPI 1640 medium supplemented with 10% FBS as described (Epperly *et al*., 2002).

Radiation survival curves

Cells were irradiated in plastic 10×100 mm test tubes at 1×10^5 cells/ml in tissue culture at a rate of 0.8 Gy/min, with doses ranging from 0 to 8 Gy. The cells were removed from the tubes and plated in semisolid medium according to published methods. The cells were incubated in a 37°C high-humidity incubator with 5% CO_2 and colonies consisting of more than 50 cells were scored on day 7. Triplicate cultures for each radiation dose were scored. Each experiment was done three times. Linear quadratic and software programs for measuring radiosensitivity and extrapolation number were used according to published methods (Epperly *et al*., 2002, 2003b).

Biochemical analysis of MnSOD activity

The nitroblue tetrazolium (NBT) reduction assay for MnSOD biochemical activity was carried out according to published methods (Epperly *et al*., 2002).

Animals and animal care

C57BL/6NHsd female mice (20 g, 6–8 weeks old; Harlan Sprague Dawley, Indianapolis, IN) were housed at five per cage according to institutional animal care and use (IACUC) protocols. Animals were irradiated with 31 Gy to the upper body, with shielding of the abdomen and head, using a linear accelerator (Varian, Palo Alto, CA) at 300 monitor units/min according to published methods (Epperly *et al*., 2003a). In other experiments total body irradiation was delivered to a dose of 9.75 Gy at a rate of 0.8 Gy/min, using a Mark IV cesium irradiator (J.L. Shepherd, Glendale, CA) according to published methods (Epperly *et al*., 2007b). All animal protocols were approved by the IACUC of the University of Pittsburgh (Pittsburgh, PA). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh.

Esophageal administration of radioprotective transgenes

C57BL/6J female mice received intraoral administration of 100 μl of water followed by full-length pNGVL3-MnSOD, blank pNGVL3 plasmid, mc-MnSOD, or control minicircle plasmid by injection of 100-μl volumes of liposomes containing 100 μg of pNGVL3-MnSOD or blank pNGVL3 blank plasmid DNA, or 50 μg of mc-MnSOD or mc-DsRed plasmid DNA according to published methods (Epperly *et al.*, 2001a, 2001b). The plasmid DNA was mixed with 28 μl of Lipofectin (Invitrogen, Carlsbad, CA) and allowed to complex at room temperature for 10 min before administration. Mice were not anesthetized, and were allowed to swallow plasmid liposome preparations. Esophageal irradiation was carried out by anesthetizing the mice with Nembutal and irradiating the esophagus to 31 Gy, using a linear accelerator according to previously published procedures (Stickle *et al.*, 1999). Mice were held and monitored for evidence of esophageal toxicity including weight loss, and dehydration. Animals were killed by euthanasia when weight loss was greater than 20%, according to IACUC protocols.

Total body irradiation protection

Mice received intravenous administration of 100 μl of liposomes containing either 100 μg of pNGVL3-MnSOD or pNGVL3 blank plasmid DNA or 50 μg of mc-MnSOD or mc-Blank plasmid by tail vein injection, 24 hr before irradiation. The DNA was complexed with Lipofectin as described above. A total body dose of 9.75 Gy (the $LD_{50/30}$, i.e., the dose of radiation required to kill 50% of a test cohort within 30 days) was administered 24 hr after gene therapy, using a Shepherd Mark IV cesium irradiator according to published methods (Epperly *et al.*, 2007b). Mice were monitored for the development of hematologic distress and were killed when moribund, or when demonstrating greater than 20% weight loss, according to IACUC protocols.

Results

Production of minicircle MnSOD plasmid

Minicircle MnSOD or DsRed plasmids were constructed by inserting the MnSOD expression cassette containing the human MnSOD transgene, CMV promoter, and poly(A) tail into the p2ϕC31 plasmid (Fig. 1A). The minicircle MnSOD plasmid was isolated and the gene sequence was obtained, indicating that the minicircle contained 54 bp from the p2ϕC31 plasmid, the CMV promoter, the human MnSOD transgene, and the poly(A) tail (Fig. 1B). A control plasmid containing the CMV promoter, DsRed transgene, and poly(A) tail was also constructed and sequenced (Fig. 1B).

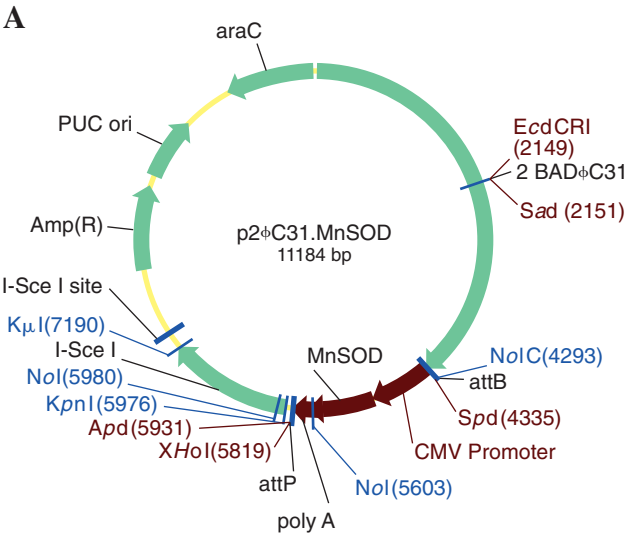

FIG. 1. Minicircle (mc) MnSOD plasmid map. The human MnSOD transgene was excised from the pNGVL3-MnSOD plasmid by PCR, using primers specific for the human MnSOD transgene, and inserted into the p2ϕC31.MnSOD plasmid (Chen *et al.*, 2005). (**A**) A map of the plasmid. (**B**) The minicircle p2ϕC31.MnSOD or minicircle DsRed plasmid was isolated and sequenced, demonstrating the presence of 54 bp of the original plasmid plus the CMV promoter, the human MnSOD or DsRed transgene, and the poly(A) sequence. The minicircle plasmids were electroporated into 32D cl 3 cells, selected for growth in G418, and cloned.

B

Minicircle Sequence (1536 bp)

Sequence from p2ϕC31 (54bp)

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CTCGAGGGGG GGCCCGCCCC AACTGGGGTA ACCTTTGGGC TCCCCGGGCG CGAC
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Sequence from CMV promoter (596bp)

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  1 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG
 61 CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT
121 GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA
181 ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
241 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA
301 CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC
361 CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG
421 ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG
481 GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
541 ACGGTGGGAG GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGC
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Sequence from MnSOD (669bp)

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  1 ATGTTGAGCC GGGCAGTGTG CGGCACCAGC AGGCAGCTGG CTCCGGCTTT GGGGTATCTG
 61 GGCTCCAGGC AGAAGCACAG CCTCCCCGAC CTGCCCTACG ACTACGGCGC CCTGGAACCT
121 CACATCAACG CGCAGATCAT GCAGCTGCAC CACAGCAAGC ACCACGCGGC CTACGTGAAC
181 AACCTGAACG TCACCGAGGA GAAGTACCAG GAGGCGTTGG CCAAGGGAGA TGTTACAGCC
241 CAGATAGCTC TTCAGCCTGC ACTGAAGTTC AATGGTGGTG GTCATATCAA TCATAGCATT
301 TTCTGGACAA ACCTCAGCCC TAACGGTGGT GGAGAACCCA AAGGGGAGTT GCTGGAAGCC
361 ATCAAACGTG ACTTTGGTTC CTTTGACAAG TTTAAGGAGA AGCTGACGGC TGCATCTGTT
421 GGTGTCCAAG GCTCAGGTTG GGGTTGGCTT GGTTTCAATA AGCAACGGGG ACACTTACAA
481 ATTGCTGCTT GTCCAAATCA GGATCCACTG CAAGGAACAA CAGGCCTTAT TCCACTGCTG
541 GGGATTGATG TGTGGGAGCA CGCTTACTAC CTTCAGTATA AAAATGTCAG GCCTGATTAT
601 CTAAAAGCTA TTTGGAATGT AATCAACTGG GAGAATGTAA CTGAAAGATA CATGGCTTGC
661 AAAAAGTAA
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Sequence from PolyA (217bp)

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  1 GCGGCCGCGA CTCTAGATCA TAATCAGCCA TACCACATTT GTAGAGGTTT TACTTGCTTT
 61 AAAAAACCTC CCACACCTCC CCCTGAACCT GAAACATAAA ATGAATGCAA TTGTTGTTGT
121 TAACTTGTTT ATTGCAGCTT ATAATGGTTA CAAATAAAGC AATAGCATCA CAAATTTCAC
181 AAATAAAGCA TTTTTTTCAC TGCATTCTAG TTGTGGT
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DsRED Sequence (Promoter to PolyA)

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  1 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG
 61 CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT
121 GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA
181 ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
241 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA
301 CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC
361 CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG
421 ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG
481 GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
541 ACGGTGGGAG GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGCGCTA
601 CCGGACTCAG ATCCACCGGT CGCCACCATG GCCTCCTCCG AGGACGTCAT CAAGGAGTTC
661 ATGCGCTTCA AGGTGCGCAT GGAGGGCTCC GTGAACGGCC ACGAGTTCGA GATCGAGGGC
721 GAGGGCGAGG GCCGCCCCTA CGAGGGCACC CAGACCGCCA AGCTGAAGGT GACCAAGGGC
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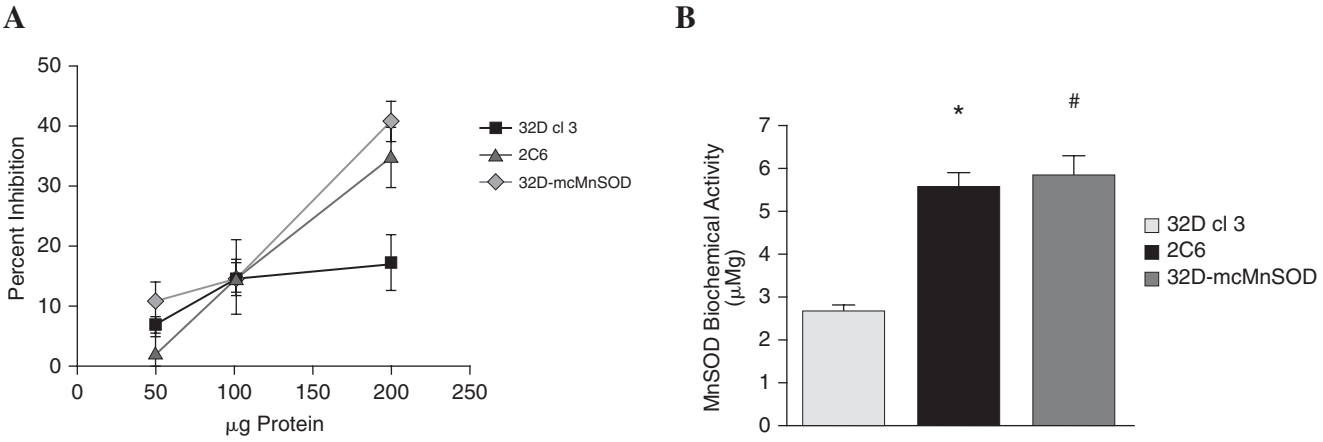

FIG. 2. Increased MnSOD biochemical activity in mc-MnSOD-transfected cells. MnSOD biochemical activity was determined in 32D cl 3, 32D mc-MnSOD, and 2C6 cell packs of 5×10^6 cells. The analysis is based on an inhibition assay in which increasing amounts of MnSOD inhibit the reduction of NBT by superoxide. (**A**) 32D mc-MnSOD and 2C6 cells presented increased inhibition of the reduction of NBT. (**B**) Inhibition of NBT reduction was converted into units of MnSOD biochemical activity per milligram of protein. One unit of activity results in 50% inhibition of NBT reduction per milligram of protein. 32D-mc-SOD and 2C6 cells had increased SOD biochemical activity levels of 5.8 ± 0.5 and 5.6 ± 0.3 U/mg, respectively, compared with 2.7 ± 0.1 U/mg for 32D cl 3 cells ($^{\#}p = 0.0039$ and $^{*}p = 0.0008$, respectively).

Transfection of 32D cl 3 cells

32D cl 3 cells were cotransfected with either minicircle MnSOD plasmid or minicircle DsRed plasmid and pSV2-neo plasmid by electroporation and selected for cells exhibiting Neo resistance. The cells were cloned by limiting dilution and RT-PCR was performed with primers specific for the MnSOD or DsRed transgene to identify clones expressing either the MnSOD or DsRed transgene. Isolation of the minicircle MnSOD plasmid yielded 0.25 to 0.5 mg of 1532-bp minicircle DNA from 1.0 liter of overnight bacterial growth. The DNA purity was about 70 to 80%, compared with the 95% purity reported by Chen and coworkers (2005).

MnSOD biochemical activity

MnSOD biochemical activity was determined in 32D cl 3, 2C6, and 32D-mc-SOD cells. Cells were lysed by repeated freeze–thaw cycles with protein concentrations measured. Using an assay in which the reaction between xanthine and xanthine oxide liberates superoxide, which then reduces NBT, resulting in an increase in color change (Epperly *et al.*, 2002). The presence of SOD removes the superoxide, preventing the color change caused by the NBT reduction. Higher concentrations of SOD will result in increased inhibition of the NBT reduction. One unit of SOD activity will result in 50% inhibition of NBT reduction per milligram of protein. Both 32D-mc-MnSOD and 2C6 had increased MnSOD activity, demonstrated by the increased inhibition of NBT color production (Fig. 2A). 32D cl 3 cells had an MnSOD biochemical activity level of 2.7 ± 0.1 U/mg protein compared with 5.6 ± 0.3 or 5.6 ± 0.5 U/mg protein for 2C6 and 32D mc-SOD cells, respectively ($p = 0.0008$ and 0.0039, respectively) (Fig. 2B).

Irradiation survival curves

To demonstrate that 32D mc-MnSOD cells had increased radiation resistance, 32D cl 3, 2C6, and 32D mc-MnSOD cells were irradiated with doses ranging from 0 to 8 Gy, plated in methylcellulose, and incubated for 7 days at 37°C in a 5% CO_2 incubator. Seven days later colonies consisting of more than 50 cells were counted and the data were analyzed in a linear quadratic model (Fig. 3). Cell lines 2C6 and 32D mc-MnSOD were more radioresistant as demonstrated by an

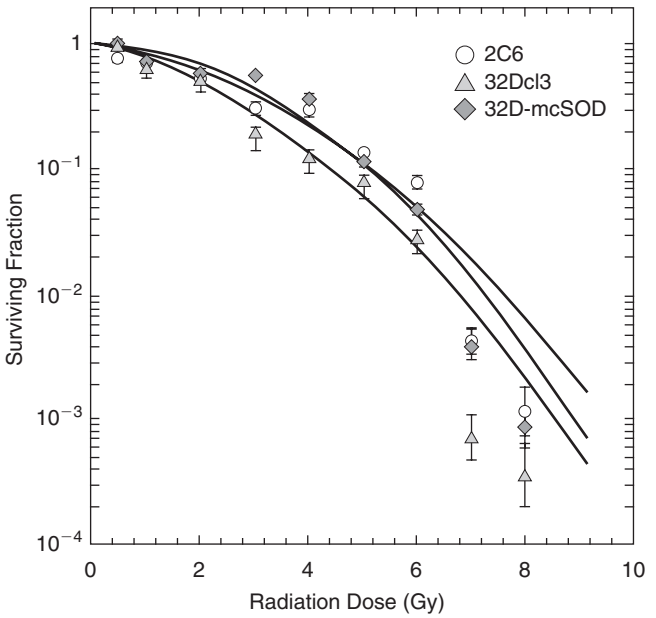

FIG. 3. Increased radioresistance of mc-MnSOD-transfected cells. Irradiation survival curves were produced for 32D cl 3, 32D mc-MnSOD, and 2C6 cell lines. Cells from either 32D mc-MnSOD or 2C6 were more resistant to irradiation and demonstrated an increased shoulder on the survival curve ($\bar{n} = 4.8 \pm 0.2$ and $\bar{n} = 4.6 \pm 0.2$, respectively) compared with $\bar{n} = 1.5 \pm 0.5$ for 32D cl 3 cells ($p = 0.0078$ and $p = 0.0070$, respectively).

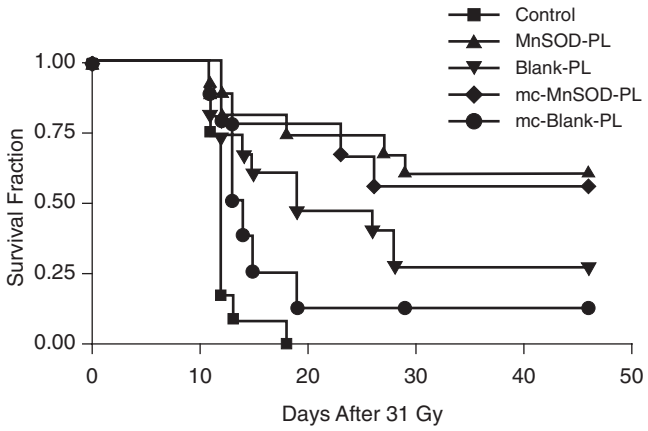

FIG. 4. Intraesophageal administration of mc-MnSOD-PL protects the murine esophagus from ionizing irradiation damage. C57BL/6NHsd female mice received intraesophageal administration of MnSOD-PL, mc-MnSOD, pNGVL3-PL, or mc-Blank (DS-Red)-PL; were then irradiated 24 hr later, along with control mice, by administration of 31 Gy to the esophagus; and were monitored for the development of severe esophagitis and weight loss (Stickle *et al*., 1999), at which time the mice were killed. Mice treated with mc-MnSOD-PL or MnSOD-PL had significantly increased survival after irradiation ($p = 0.0003$ or $p < 0.0001$, respectively) compared with control irradiated mice. Administration of blank full-length plasmid containing bacterial CpG sequences pNGVL3-PL resulted in increased survival ($p = 0.0012$). In contrast, control mc-Blank (DS-Red)-PL produced no change in survival. There was a statistically significant increase in survival when comparing mc-MnSOD-PL with mc-Blank-PL ($p = 0.0391$).

increase in the shoulder on the survival curve, with $\overline{n} = 4.6 \pm 0.2$ and $\overline{n} = 4.8 \pm 0.2$ for 2C6 and 32D mc-MnSOD cells, respectively, compared with $\overline{n} = 1.5 \pm 0.5$ for 32D cl 3 cells ($p = 0.0070$ and 0.0078, respectively). There was no significant change in D_0 between the cell lines.

In vivo *esophageal radioprotection*

We had previously demonstrated that intraesophageal administration of MnSOD-PL protects the esophagus from irradiation damage (Stickle *et al*., 1999; Epperly *et al*., 2001). To determine whether mc-MnSOD was similarly radioprotective, 100 μg of pNGVL3 MnSOD plasmid or blank pNGVL3 DNA was complexed with 28 μl of lipofectant and 50 μg of mc-MnSOD plasmid or DsRed plasmid was mixed with 28 μl of lipofectant and incubated for 10 min at room temperature. The plasmid–liposome complexes were each administered by plastic syringe to the top of the esophagus of nonanesthetized C57BL/6NHsd female mice (15 per group) and the mice were allowed to swallow the complexes as described (Stickle *et al*., 1999). The PL-treated mice as well as untreated control mice were then irradiated 24 hr later by administration of 31 Gy to the esophagus and the mice were monitored for the development of esophagitis (Fig. 4). Mice swallowing either mc-MnSOD-PL or MnSOD-PL had improved survival after irradiation compared with the irradiated control mice ($p = 0.0003$ and $p < 0.0001$, respectively). Mice given pNGVL3-PL had some detectable increased survival compared with the control irradiated mice ($p = 0.0012$), but there was further improved survival among those given MnSOD-PL. In contrast, there was no significant difference in survival between control irradiated mice and mice injected with control minicircle DsRed-PL. The mice given mc-MnSOD-PL had significantly increased survival compared with control minicircle Blank DsRed-PL-treated mice ($p = 0.0391$).

In vivo *radiation protection against total body irradiation*

To demonstrate that overexpression of MnSOD in a pNGVL3 or minicircle plasmid could be safely administered systemically, C57BL/6NHsd female mice (15 mice per group) were injected intravenously via the tail vein with MnSOD-PL, mc-MnSOD-PL, pNGVL3 plasmid, or mc-Blank-PL, and irradiated 24 hr later along with control mice to 9.75 Gy whole body irradiation. The mice were monitored for signs of hematologic stress, at which time they were killed (Fig. 5). mc-MnSOD-PL as well as full-length MnSOD-PL provided significant protection from 9.75 Gy total body irradiation ($p < 0.0001$ and $p = 0.0340$, respectively).

Discussion

The development of systemic protection agents against total body ionizing irradiation is a focus of great scientific interest (Stone *et al*., 2004). Normal tissue-specific radioprotection is highly desirable in the development of new chemoradiotherapy programs for clinical cancer patient management (Greenberger *et al*., 2003; Greenberger and Epperly, 2004). Furthermore, normal tissue-specific radioprotection in the total body-irradiated patient has been the subject of particular interest particularly since solid tumors have been demonstrated to have a different redox balance compared with normal tissues (Spitz *et al*., 2004). Organ-specific systemic radioprotector agents have included strategies to neutralize inflammatory cytokines using small molecule antioxidants, by targeting the apoptotic cellular mechanism of death by administration of agents that upregulate antiapoptotic proteins, or neutralize proapoptotic proteins (Green-

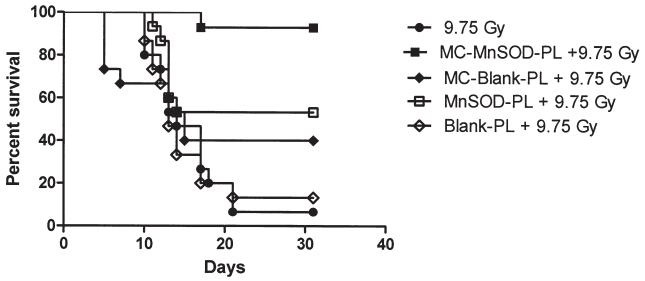

FIG. 5. Systemic delivery of mc-MnSOD-PL or MnSOD-PL protects against whole body irradiation. Mice were injected intravenously with 100 μl of liposomes containing 50 μg of plasmid DNA (mc-MnSOD-PL and mc-Blank-PL), or 100 μg of plasmid DNA for MnSOD-PL or pNGVL3-PL (Blank-PL) and irradiated to 9.75 Gy total body irradiation 24 hr later. The mice were monitored for the development of hematologic stress, at which time they were killed. Mice given mc-MnSOD-PL or MnSOD-PL were significantly protected compared with irradiated controls ($p < 0.0001$ and $p = 0.0340$, respectively). Mice injected with mc-Blank-PL or Blank-PL were not significantly different from control 9.75 Gy-irradiated mice.

berger and Epperly, 2007). Each of these strategies has provided some success. However, management of side effects, and incomplete systemic delivery, have been complicating factors.

One approach toward radiation protection has focused on the cellular and tissue redox balance mechanisms and their alteration by ionizing irradiation (Epperly *et al*., 2004; Greenberger *et al*., 2003). Antioxidant stores within cells include glutathione, and other free radical-scavenging antioxidants. Prominent in this cellular arsenal are the antioxidant enzymes including superoxide dismutases (Hahn *et al*., 2000; Mitchell and Krishna, 2002; Spitz *et al*., 2004). Two forms of copper/zinc metalloenzyme, one intracellular and another extracellular, are involved in systemic protection against agents that induce oxidative stress, including tissue inflammation induced by ionizing irradiation (Spitz *et al*., 2004). Manganese superoxide dismutase, the third form of SOD, targets the mitochondria by a 22-amino acid targeting sequence (Epperly *et al*., 2003b). Specific localization of SOD to the mitochondria appears necessary for radiation protection of cells *in vitro* and tissues *in vivo* because removal of the mitochondrial localization sequence produces MnSOD with cytoplasmic expression and little radioprotective capacity, whereas attaching this same mitochondrial localization signal to copper/zinc SOD results in a mitochondria-targeted and radioprotective copper/zinc metalloenzyme (Epperly *et al*., 2003b).

Administration of MnSOD protein to cells in culture or tissues *in vivo* has not shown significant radioprotective capacity, in part because of difficulty in achieving high enough levels of protein at the mitochondrial membrane, where critical antioxidant function is required (Epperly *et al*., 2001). In contrast, administration of transgene cDNA for MnSOD results in transport to the mitochondria at high efficiency of mitochondria-targeted protein and has been shown to confer radioprotection to cells *in vitro* and tissues *in vivo* (Stickle *et al*., 1999; Epperly *et al*., 2002, 2003b, 2003c; Carpenter *et al*., 2005).

For systemic radiation protection, intravenous administration of plasmid liposomes has been shown to induce inflammation (Sellens *et al*., 2005), and this has been attributed to CpG sequences in the bacterial backbone of the plasmid (Sellens *et al*., 2005). An important strategy toward eliminating the negative side effects of plasmid liposome gene therapy has been reported by demonstration of a technique by which to construct a minicircle plasmid devoid of bacterial CpG sequences (Chen *et al*., 2005).

In the present study, we used this published technique of construction of minicircle plasmid to produce minicircle MnSOD, demonstrating its effectiveness in transferring human MnSOD transgene-mediated biochemical activity to mice, hematopoietic progenitor cells *in vitro*, and the esophagus of mice *in vivo*. Furthermore, intravenous administration of minicircle MnSOD was associated with protection from the $LD_{50/30}$ total body irradiation dose of 9.75 Gy.

Previous studies demonstrated some radioprotective capacity of empty full-length plasmid, PNGVL3 or PRK5 (Epperly *et al*., 1999). This protection was associated with an inflammatory response that could be detected in tissues as an increase in mRNA for tumor necrosis factor-α, IL-1, and transforming growth factor-β (Epperly *et al*., 2001b). Although a nonspecific inflammatory response may be radioprotective in some organ-specific settings, there is concern that systemic introduction of a gene therapy vector that itself is inflammatory might be deleterious in a setting of victims of an irradiation accident or willful irradiation terrorism event, wherein the ionizing irradiation itself and combined injury (heat, physical trauma) would be inflammatory. The present mc-MnSOD gene therapy vehicle should eliminate inflammatory side effects of the vector but maintain sufficient radioprotective capacity to allow its use by intravenous administration in larger mammalian species including humans.

Preparation of mc-MnSOD constructs was not a trivial process. The techniques for bacterial growth, centrifugation, purification, and extraction of minicircle plasmid were complex. Furthermore, previous studies demonstrated yield of minicircle as high as 96% of total recovered plasmid liposome (Chen *et al*., 2005), whereas in the present study yield rates as low as 68% were noted. These differences were not attributable to obvious changes in laboratory technique, bacterial expression system, centrifugation technique, or extraction methods. It is possible that the MnSOD transgene or other antioxidant transgenes yet to be tested may, as part of the insertion and recombination process, result in a lower yield of minicircle construct. Further studies will be required to increase the percent yield and quantity of mc-MnSOD construct for gene therapy experiments in primates and for clinical trials in patients.

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Author Disclosure Statement

No competing financial interests exist.

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