in vivo *19*: 997-1004 (2005)

MnSOD-Plasmid Liposome Gene Therapy Decreases Ionizing Irradiation-Induced Lipid Peroxidation of the Esophagus

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Abstract. *Background: Ionizing irradiation-induced cellular and tissue damage is mediated in part by resultant radiochemical reactions and resultant oxidative stress. Irradiation-induced reactive oxygen and nitrogen species include: superoxide, nitric oxide, hydroxyl radical and hydrogen peroxide. The biochemical combination of superoxide and nitric oxide radicals forms peroxynitrite, a potent oxidant known to induce lipid peroxidation. Materials and Methods: The antioxidant capacity and lipid peroxidation of the esophagus were determined following irradiation. Results: In the present studies, measurements of total antioxidant capacity did not change in the esophagus of control irradiated or control plasmid pNGVL3-PL intraesophageally-injected mice. In contrast, manganese superoxide dismutase-plasmid/liposome (MnSOD-PL) intraesophageally-treated mice showed a significant increase in antioxidant capacity persisting for seven days. Lipid peroxidative changes induced in the control irradiated mouse esophagus decreased over seven days after irradiation of C3H/HeNHsd mice exposed to 37 Gy in a single fraction. MnSOD-PL radioprotective gene therapy administered intraorally 24 hours prior to irradiation did not significantly reduce the kinetics of induction of total peroxidated lipids over the first seven days after irradiation but did decrease lipid peroxidation at days 14 and 21. Conclusion: These studies demonstrate the antioxidant function of MnSOD-PL gene therapy to the esophagus, which is detectable as a reduction in irradiation-induced lipid peroxidation.*

Cellular and tissue damage induced by ionizing irradiation has been shown to involve biochemical steps at the molecular, intracellular and intercellular levels which are common to a range of other inflammatory stimuli including: hypoxia, ischemia reperfusion injury and toxic chemical damage (1-9). In particular, ionizing irradiation-induced free radicals and oxidants (including superoxide, nitric oxide (10), hydroxyl radical, hydrogen peroxide and peroxynitrite) mediate cellular damage (11, 12) *via* the process of lipid peroxidation (13). Oxidative lipid damage to the nuclear membrane, but also the mitochondrial and cell membrane (14), have been shown to include peroxidative changes. Specific lipid peroxide moieties, formed in ionizing irradiated cells *in vitro* and in tissues *in vivo,* have been shown to be common in molecular structure and distribution to lipid peroxidative changes that are induced by inflammatory cytokines (15-21), ischemia reperfusion injury (2) and by toxic chemicals, and which are associated with the induction of apoptosis through mitochondrial cytochrome C release (15-17) and activation of caspase pathways leading to nuclear DNA fragmentation (11, 12).

Ionizing irradiation-induced tissue damage is known to be directly related to total irradiation dose, irradiation fraction size and volume of tissue irradiated (22). Depletion of antioxidant reserves within cells and tissues increases ionizing irradiation injury while restoration of antioxidant levels, including thiols and glutathione, decrease irradiation-induced cell and tissue death (23, 24). Direct irradiation dose-response relationships to redox imbalance within cells and tissues have been shown to correlate to lipid peroxidative changes (13-15).

We have previously demonstrated that delivery of intraoral (swallowed) MnSOD-PL gene therapy to mice provides significant protection against single fraction (25) or fractionated ionizing irradiation damage to the esophagus (26, 27). With cells in culture, MnSOD-transgene-mediated ionizing irradiation protection has been shown to decrease the formation of specific lipid peroxidative changes in the mitochondrial membrane (14, 15, 24). In the present studies, we sought to determine whether irradiation protection of the murine esophagus by MnSOD-PL gene therapy correlated with *in vivo* detectable decreases in tissue lipid peroxidation.

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Key Words: Lipid peroxidation, esophagus, radioprotection, MnSOD-Plasmid Liposomes, gene therapy.

Materials and Methods

Mice, plasmid/liposome and irradiation. Plasmid/liposome complexes were prepared by mixing 11 μl of lipofectant (Gibco BRL, Gaithersburg, MD, USA) with 200 μg of plasmid DNA in a final volume of 100 μl of PBS, incubated at room temperature for 30 min. Male C3H/H3NHsd mice (Harlan Sprague Dawley, Indianapolis, Ind., USA), 10-12 weeks of age, were treated by placing a feeding tube attached to a 1 cm syringe through the oral cavity and injecting 100 μl of water followed by 100 μl of plasmid/liposome complex into the top of the esophagus. The material was swallowed (25). Groups of mice were injected 24 h preirradiation with liposome complexes containing: i) no plasmid DNA, ii) control pNGVL3 plasmid DNA, or iii) pNGVL3-MnSOD plasmid DNA containing the human MnSOD transgene (27). The mice were then irradiated to 37 Gy (Varian 6 MeV linear accelerator, 200 cGy/min dose-rate). The mice were shielded, as published (25, 55, 56), so that only the pulmonary cavity was irradiated. The abdomen, head and extremities remained outside the field of irradiation.

Biodistribution of intraesophageally-administered MnSOD-PL transcripts. C3H/HeNHsd mice (5 males and 5 females per group) received intraesophageal administration of water, pNGVL3-PL or MnSOD-PL, and were sacrificed 24 h later. Parts of the following tissue were removed: ovaries, skin, muscle, bone marrow, blood, heart, brain, liver, kidney, urine, bladder, rectum, feces, intestines, stomach, lungs, trachea, esophagus and tongue, and frozen in liquid nitrogen. DNA was extracted using a DNeasy 96 Tissue Kit by Qiagen (Valencia, CA, USA). Polymerase chain reaction (PCR) was performed using primers specific for the human MnSOD transgene and primers for mouse actin to demonstrate that the PCR reaction occurred properly. Electrophoresis of the PCR products was carried out in 1% agarose gel and stained with ethidium bromide.

Measurement of cytokine transcript levels in the esophagus by RNase protection assay. C3H/HeNsd mice were injected intratracheally with clinical-grade MnSOD-PL, pNGVL3-PL or liposomes containing no DNA. The mice were irradiated to the pulmonary cavity with a dose of 37 Gy. The mice were killed humanely 0, 1, 4 or 7 days later; the esophagus was removed and snap-frozen in liquid nitrogen, and RNA was extracted using Triazol. Cytokine expression was analyzed using RiboQuant RNase Protection Kit Multi-Probe Template Sets (mCK-2b and mCK-3b) (Pharmingen, San Diego, CA, USA). Densitometry was determined using a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). The results were normalized against the internal standards L32 and glyceraldehydes-3-phosphate dehydrogenase (GAPD) provided with the assays. Cytokine mRNA levels measured included TGF-β1, TGF-β3, TNF-α, TNF-β, Ltb, IFNg, INFb, Mif, IL-1a, IL-1B, IL-1ra, IL6, IFNg, IL10, IL12 and interferon gamma inducing factor (IGIF, now known as Ifngr) (27).

Measurement of antioxidant capacity of the esophagus following irradiation. Male C3H/HeNHsd mice were treated with water alone, pNGVL3-PL or MnSOD-PL, as described above, followed by irradiation to 37 Gy to the esophagus 24 h later. The mice were sacrificed at 0, 1, 2, or 7 days after irradiation. The esophagus was removed, frozen in liquid nitrogen, homogenized and the antioxidant capacity was measured using an antioxidant reductive capacity assay (Northwest Life Science Specialties, LLC, Vancouver, WA, USA). Following homogenization, the protein was quantitated and 100 μg of protein was diluted into the assay dilution buffer for a final volume of 800 μl. Two hundred μl of the diluted samples were placed in each of 3 wells, and the plate was read at 490 nm. To each well was added 50 μl of Cu+/+ solution and incubated for 3 min at room temperature and stopped by the addition of 50 μl of the stop solution, and the plate was then read a second time at 490 nm. By plotting the difference between the two absorbance readings and comparing them to a standard curve, we quantitated the antioxidant capacity of the solutions.

Measurements of lipid peroxidation. Two methods were used to analyze lipid peroxidation following irradiation. In the first assay, control mice as well as mice that had been administered MnSOD-PL 24 h earlier were sacrificed and the esophagus was removed, prepared as a single cell suspension by teasing the tissue in a solution containing 0.2% type XY collagenase, dispase (grade 2, 240 units) and 0.1% trypsin, and incubated for 1 h at 37˚C. The cell suspensions and DMEM were drawn through proportionately smaller gauge needles to a 27-gauge needle and were then filtered through 100 μM and 45 μM filters to remove cell clumps. The cells were stained with anti-CD45-FITC and anti-Ter119-PC7 and sorted by flow cytometer (55, 56) to remove the hematopoietic cells from the esophageal cells. The cells were then irradiated to 10 Gy and plated in T25 flask in DMEM media containing 10% FCS. Twenty-four h later, the cells were removed from the flask, pelleted and frozen at –80˚C. The cells were thawed and the lipid peroxidation measured using a Malondialdehyde assay (Northwest Life Science Specialties). The cells were resuspended in 250 μl of the calibrator buffer to which was added 10 μl of BHT reagent, 250 μl of the acid reagent and 250 μl of TBA reagent. This was vortexed and then incubated for 60 min at 60˚C and centrifuged at 10,000 X g for 3 min. The supernatant was then placed in a cuvet which was read at 520 nm.

As a second method, measurement of *in vivo* lipid hydroperoxide (57) was carried out. C3H/HeNHsd male mice were injected intraesophageally with pNGVL3-PL or MnSOD-PL and irradiated 24 h later to 37 Gy to the pulmonary cavity. The mice were sacrificed at days 0, 1, 2, 3, 7, 14 or 21 after irradiation (3 mice per group). The esophagus was removed and frozen in liquid nitrogen with the lipids extracted and the amount of lipid phosphorous determined using a micro-method. Total lipid extracts were obtained by the Folch procedure. The esophagus was homogenized in methanol and mixed with 1 ml of chloroform and kept under nitrogen for 1 h on ice (in the dark) then 0.3 ml of 0.1 M sodium chloride was added and the mixture was vigorously vortexed under nitrogen. The chloroform layer was separated by centrifugation and chloroform was dried under the steam of nitrogen. Lipids were dissolved at 100 μl of hexane-isopropanol-water (3-4-0.5 v/v mixture).

Lipid hydroperoxides were determined by fluorescence HPLC of products formed in the peroxidase-catalyzed reaction of specific lipid hydroperoxides with a fluorogenic substrate, Amplex Red. HPLC separation of the reaction mixture with fluorescent detection of resorufin (an Amplex Red oxidation product) is conducted as follows. The assay is started by the addition of 1 μL of reaction mixture containing 50 μM Amplex Red to 100 μL of basic reaction

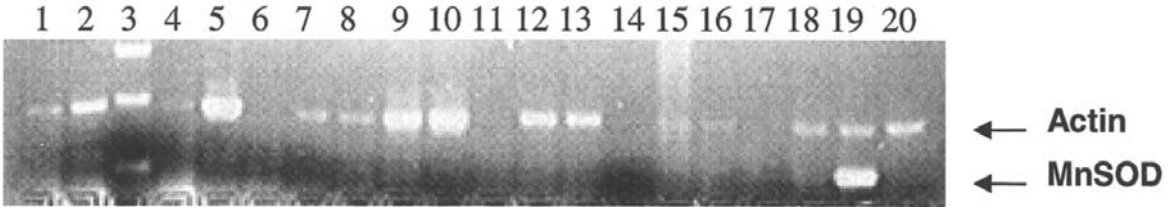

Figure 1. *Biodistribution of MnSOD transgene transcripts following intraesophageal injection of MnSOD-PL. Groups of at least 5 male and female C3H/HeNHsd mice were injected intraesophageally with water, pNGVL3-PL or MnSOD-PL (200 μg plasmid DNA) and sacrificed 24 h later. Tissue samples from the major organs were collected and frozen in liquid nitrogen. DNA was extracted, and PCR performed using primers specific for the human MnSOD transgene or actin. The PCR products were electrophoresized and stained with ethidium bromide. Results from one representative mouse injected with MnSOD-PL are shown: lane 1 contains DNA from the ovaries, 2 skin, 3 DNA ladders, 4 muscle, 5 bone marrow, 6 blood, 7 heart, 8 brain, 9 liver, 10 kidney, 11 urine, 12 bladder, 13 rectum, 14 feces, 15 intestine, 16 stomach, 17 lungs, 18 trachea, 19 esophagus and 20 tongue. The upper band represents the actin product, the bottom band the human MnSOD product. Expression of the MnSOD transgene was identified in the esophagus (lane 19) of mice receiving MnSOD-PL. In some mice, expression was also detected in the lung and oral cavity (tongue). No expression of the human MnSOD transgene was detected in any of the tissues of mice receiving water or pNGVL3-PL.*

mixture containing 25 mM NaH_2PO_4, 0.5 mM EDTA (pH 7.4 at 4˚C) and an aliquot (1-2 μL) of lipid samples dissolved in ethanol. The reaction was initiated by addition of 1 μL of microperoxidase solution (0.25 μg/μL). The samples were incubated at 4˚C for 40 min. The reaction was terminated by addition of 100 μL of stop solution (10 mM HCl, 4 mM butylated hydroxytoluene in ethanol). The samples were centrifuged at 15,000 xg for 5 min and the supernatant was used for HPLC analysis. Aliquots (5 μL) were injected into a C-18 reverse phase column (Eclipse XDB-C18, 5 μM, 150 X 4.6 mm). The column was eluted by mobile phase composed of 25 mM NaH_2PO_4 (pH 7.0)/methanol (60:40 v/v) at 1 mL/min of flow rate. The resorufin fluorescence was measured at 590 nm after excitation at 560 nm. The Shimadzu LC-100AT vp HPLC system equipped with a fluorescence detector (model RF-10Axl) and autosampler (model SIL-10AD vp) was used. Chromatograms were processed and stored in digital form with Class-VP software.

Statistics. The data are presented as mean±SEM and significance of differences were assessed by Student's *t*-test. Differences were considered significant at $p<0.05$.

Animal assurance and welfare. All animal protocols used in these studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, USA. All procedures were performed under the supervision of the Division of Laboratory Animal Research of the University of Pittsburgh. Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. The mice were not subjected to any discomfort, distress, pain, or injury other than what has been described.

Results

Biodistribution of MnSOD-PL. To determine which tissues were detectably transfected following intraesophageal administration of MnSOD-PL, C3H/HeNHsd mice (male and female) received intraesophageal water only, pNGVL3-PL or MnSOD-PL, and were sacrificed 24 h following injection. The various tissues of the mice were excised and frozen in liquid nitrogen. The DNA was extracted and PCR performed using primers specific for the human MnSOD transgene as well as primers for actin to indicate that the PCR reaction occurred. The PCR products were run on a 1% agarose gel and stained with ethidium bromide. Figure 1 demonstrates the results obtained from a representative mouse injected with MnSOD-PL where the plasmid was located only in the esophagus. In some of the mice injected with MnSOD-PL, plasmid containing the human MnSOD transgene was detected in the lungs, trachea and tongue or oral cavity as well as the esophagus. No human MnSOD transgene was detected in any of the tissues from mice injected with water only or pNGVL3-PL. Uptake of the MnSOD-PL was similar in both male and female mice.

Administration of MnSOD-PL reduces levels of irradiation-induced inflammatory cytokine transcripts in the esophagus. C3H/HeNHsd mice which had received intraesophageal injection of liposomes only or MnSOD-PL, were irradiated to 37 Gy 24 h later and were then sacrificed on days 0, 1, 4 or 7 after irradiation. The esophagus was removed, frozen in liquid nitrogen and RNA extracted using Triazol. The RNase Protection Assay was used to measure cytokine expression for IL1β, IL1α receptor, IFNγ, IFNγ receptor, IL6, LTα, LTβ, TNFα, TGFβ and MIF in the different groups. Following irradiation, there was increased expression of several cytokines by day 7 including IL1β, IL1α receptor, IFNγ, IFNγ receptor, IL6, TNFα and TGFβ. The expressions of IFNγ and TNFα were increased in irradiated mice and those injected with liposomes alone then irradiated, but not in mice injected with MnSOD-PL prior to irradiation (Figure 2).

Antioxidant capacity of the murine irradiated esophagus is increased in mice treated with MnSOD-PL. Tissues with higher antioxidant capacity should have an increased ability

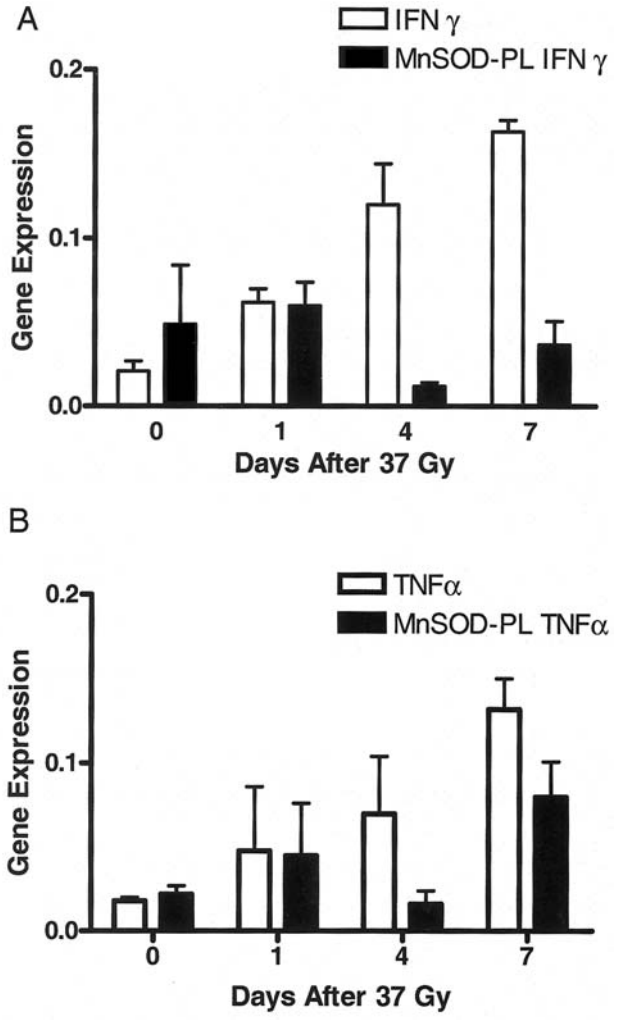

Figure 2. *MnSOD-PL treatment decreases esophageal gene expression of the transcripts for IFNγ and TNFα following 37 Gy irradiation of the esophagus. Control C3H/HeNHsd mice or mice treated with MnSOD-PL 24 h earlier were irradiated to 37 Gy to the pulmonary cavity. The mice were sacrificed on days 0, 1, 4, or 7 following irradiation. The esophagus was removed, frozen in liquid nitrogen, RNA extracted and RNase Protection Assay was performed to determine gene expression for IL1β, IL1α receptor, IFNγ, IFNγ receptor, IL6, LTα, LTβ, TNFα, TGFβ and MIF. Control irradiated mice showed significantly increased expression ($p<0.05$) of IFNγ at day 4 and 7 (Figure 2A) and TNFα at day 7 (Figure 2B) following irradiation, while MnSOD-PL-treated animals showed no significant increase in expression levels of the transcripts for these cytokines. (At least 3 mice per data-point.) There were no significant irradiation-induced changes in gene expression for the other cytokines (data not shown).*

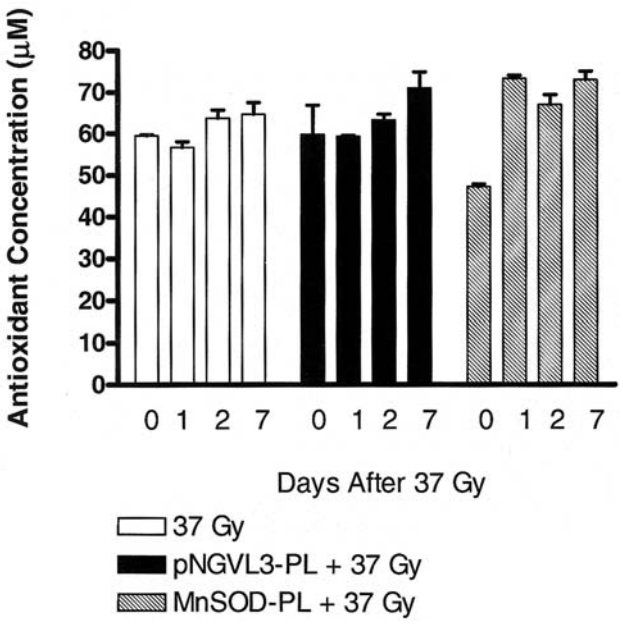

Figure 3. *Increased relative antioxidant capacity of esophageal tissue removed from MnSOD-PL-treated mice. Control irradiated C3H/HeNHsd mice and mice injected intraesophageally with either MnSOD-PL or pNGVL3-PL (200 μg plasmid DNA) were irradiated to 37 Gy 24 h later to the upper body and sacrificed on days 0, 1, 2, or 7 following irradiation. The esophagus was removed, frozen in OCT, homogenized and the total antioxidant capacity measured using an Antioxidant Reductive Capacity Assay (Northwest Life Science Specialties). Following irradiation there was no significant change relative to day 0 in antioxidant capacity in the control irradiated or pNGVL3-PL-treated mice. There was a significant increase relative to day 0 in the antioxidant capacity of the mice treated with MnSOD-PL on days 1, 2 or 7 following irradiation ($p<0.0001$, 3 mice per group).*

to reduce reactive oxygen species and scavenge radicals produced following irradiation and should show irradiation protection. Mice received water only, pNGVL3-PL, or MnSOD-PL and were irradiated to 37 Gy to the esophagus 24 h later. The esophagus was removed and the antioxidant capacity determined. Following irradiation, there was no change in the antioxidant capacity of the esophagus from mice injected with water only, or pNGVL3-PL. The antioxidant capacity of the esophagus from mice injected with MnSOD-PL had a relatively decreased antioxidant capacity at the time of irradiation (Figure 3); however, following irradiation, the antioxidant capacity of the esophagus in this group increased on day 1 and continued to be significantly elevated through day 7.

MnSOD-PL treatment reduces irradiation-induced lipid peroxidation of the esophagus. To determine whether administration of MnSOD-PL reduced irradiation-induced lipid peroxidation, we measured lipid peroxidation by two methods. The first method was used to measure lipid peroxidation following irradiation of explanted esophageal cells *in vitro*. Irradiation of the control 32D cl 3 cell line (11) was used to demonstrate the capacity of the Malondialdehyde assay (Northwest Life Science Specialties) to measure lipid peroxidation. Irradiation of 32D cl 3 cells

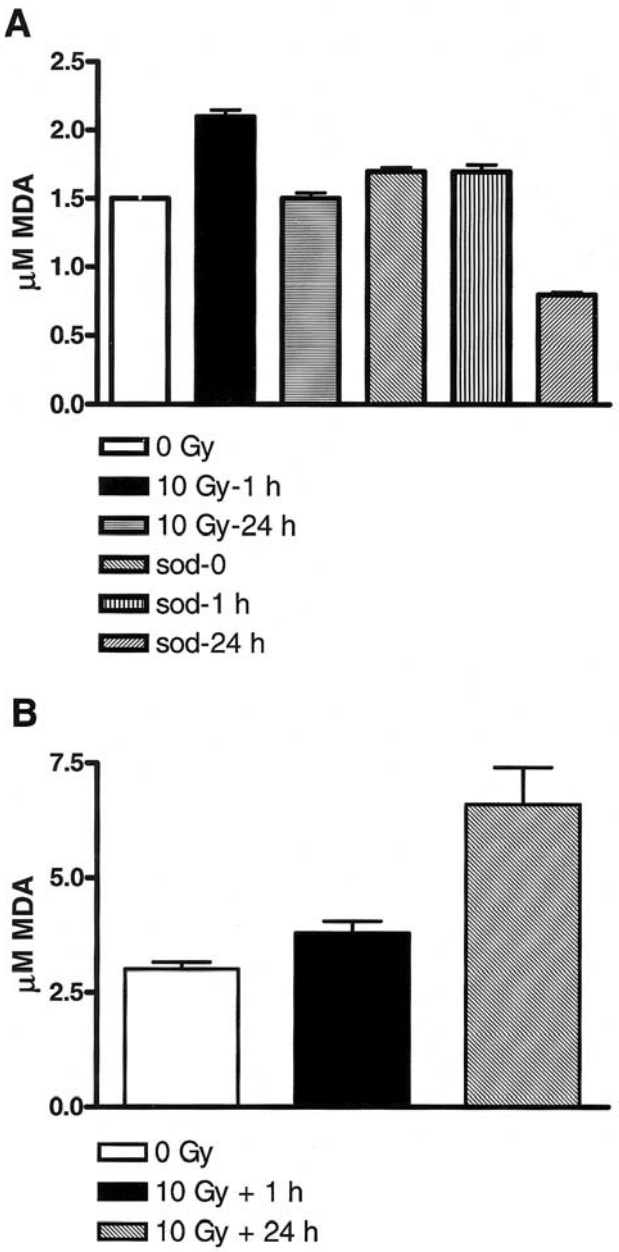

Figure 4. *Esophageal MnSOD-PL administration in vivo decreases the level of in vitro irradiation-induced lipid peroxidation. A Malondialdehyde Assay was utilized to detect lipid peroxidation. Control C3H/HeNHsd mice and mice injected intraesophageally with MnSOD-PL 24 h previously (SOD) were sacrificed. The esophagus was removed from each mouse and single cell suspensions prepared. The cells were stained with antibodies to CD45 and Ter 119 and sorted by flow cytometer (55, 56) for removal of hematopoietic cells. The isolated esophageal cells from at least 2 mice per group were irradiated to 10 Gy and lipid peroxidation measured at 1 h and 24 hrs. The irradiated esophageal cells from the control irradiated mice demonstrated increased lipid peroxidation above baseline levels by 1 h following irradiation. The increased level was not observed at 1 h after irradiation in the MnSOD-PL-treated mice (Figure 4A). As the assay control, cells from 32D cl 3 (39) were irradiated to 10 Gy and lipid peroxidation measured at 1 h and 24 h. The 32D cl 3 cells showed significantly increased lipid peroxidation at 24 h following irradiation (Figure 4B).*

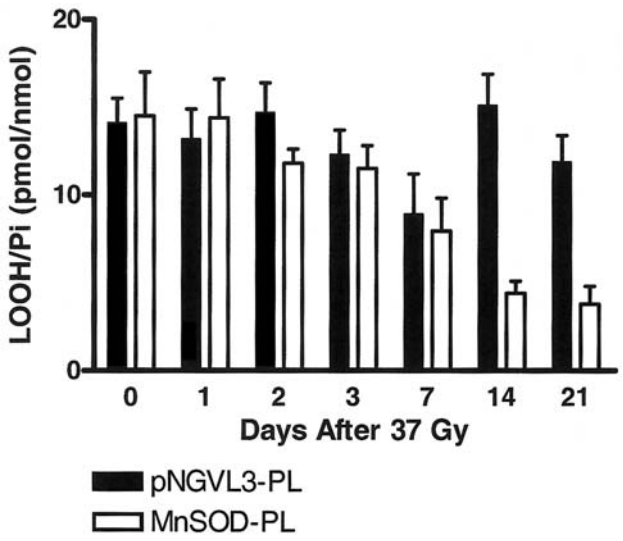

Figure 5. *Esophageal MnSOD-PL administration decreases the level of irradiation-induced lipid peroxidation in vivo. Groups of 21 C3H/HeNHsd mice were injected intraesophageally with MnSOD-PL or control pNGVL3-PL (200 μg plasmid DNA), were irradiated 24 h later to 37 Gy to the esophagus, and sacrificed at 0, 1, 2, 3, 7, 14 or 21 days later. The esophagus from 3 mice per group was removed at each time point and frozen in liquid nitrogen, homogenized, and lipid hydroperoxides measured. At days 14 and 21 there was a significant reduction of lipid hydroperoxides relative to prior levels in the mice treated with MnSOD-PL ($p=0.0363$ or 0.0293, respectively), but not in the pNGVL3-PL treated mice.*

to 10 Gy resulted in increased lipid peroxidation at 1 and 24 h after irradiation (Figure 4B). Esophageal cells were isolated from explanted tissues from mice that had been injected with water only, or MnSOD-PL and the cells were then irradiated *in vitro* to 10 Gy. Lipid peroxidation was measured at 1 h and 24 h after *in vitro* irradiation. There was a clear increase in peroxidation of lipids detected 1 h after irradiation in the control water-injected animals. In contrast, the level of peroxidized lipids in the irradiated esophagus was decreased prior to MnSOD-PL treatment (Figure 4A).

As a second method to measure lipid peroxidation, we carried out MnSOD-PL treatment and *in vivo* irradiation. Groups of mice injected with pNGVL3-PL or MnSOD-PL 24 h earlier were tested for *in vivo* changes detected at 0, 1, 2, 3, 4, 7, 14 or 21 days after irradiation. The mice were the sacrificed, the esophagus removed and lipid hydroperoxides measured (Figure 5). At day 7 after irradiation, lipid hydroperoxides were decreased in mice that received pNGVL3-PL or MnSOD-PL. However, at day 14 and 21, there was a secondary increase in lipid peroxidation in the esophagus of mice treated with pNGVL3-PL, not observed in the MnSOD-PL-treated mice (Figure 5).

Discussion

Ionizing irradiation-mediated cellular and tissue damage in the esophagus is known to include biochemical pathways common to multiple other forms of tissue injury including hypoxia (6), ultraviolet irradiation (1), inflammatory cytokines (2, 3), and inflammatory cellular-mediated tissue damage (8, 9). The common element of lipid peroxidation in these several forms of tissue damage is consistent with the capacity of each of these damage-inducing agents to induce oxidative stress which is mediated by free radical production (24). Depletion of cellular and tissue antioxidant stores, notably depletion of thiols and glutathione, is known to increase the baseline levels of lipid peroxidation (39). Lipid peroxidative changes are known to mediate membrane permeability changes associated with mitochondrial membrane permeability and leakage of cytochrome C (11, 12). Cytochrome C is a common mediator of caspase activation and nuclear DNA fragmentation associated with apoptosis (17). Thus, lipid peroxidative changes in the mitochondrial membrane have been shown to be related to the pathway of apoptotic cell death.

The present studies demonstrated that ionizing irradiation induces lipid peroxidation in a murine esophagus and that this level is decreased by treatment with MnSOD-PL. We demonstrated increased expression of IFNg at 4 and 7 days after irradiation and increased TNF-α at day 7 in the mouse esophagus following 37 Gy in mice treated with control plasmid pNGVL3-PL. This induction was not seen in MnSOD-PL-treated mice (27). The lipid peroxidation at day 7 may be attributable to synthesis of new lipids during cell division required to replace damaged cells. By day 14, in the control irradiated or the pNGVL3-PL-treated mice, the increase in cytokines may have been responsible for the cell division and a delayed second wave of oxidant-mediated increase in lipid peroxidation. Thus, our method of MnSOD-PL treatment decreased both cytokine expression and lipid peroxidation.

The lipid peroxidation we observed *in vivo* was probably not directly induced by irradiation. Instead, lipid peroxidation was most likely caused by an inflammatory response triggered by irradiation. Therefore, lipid peroxidation and increased pro-inflammatory cytokines were likely to correlate (which seemed to be true in the present data). Our methods used in Figure 5 were particularly good for *in vivo* assays of lipid peroxidation. In contrast, the MDA measurements (Figure 4) for *in vitro* irradiation were not applicable *in vivo*. Thus, we needed two methods for the *in vitro* and *in vivo* assays of esophageal lipid peroxidation. Both methods proved valuable and the results correlated to some extent. Lipid peroxidation is known to occur after some significant depletion of antioxidant reserves. This time lag in part explains the observed time course of changes in antioxidant reserves relative to the increases in lipid peroxidation.

The most likely mechanism of MnSOD-PL-mediated reduction of irradiation-induced lipid peroxidative damage in the esophagus was through stabilization of antioxidant pools and neutralization of superoxide by MnSOD enzyme action. In support of this hypothesis is other data showing that adding small molecule antioxidant radioprotectors to cells in culture also reduced the radiation-induced lipid peroxidation (41, 42, 53). Another possibility is that the lipid in the liposomes themselves could have stabilized the cell membrane, or mitochondrial membrane lipids at molecular target sites of lipid peroxidation. Against this latter possibility is the data showing that control groups receiving empty liposomes had less significant radioprotection. The data suggest that the MnSOD transgene product in large part mediated protection. The data also suggest that, following irradiation, increased expression of MnSOD results in a significantly increase antioxidant capacity that may be responsible for the reduction of lipid peroxidation detected in these studies.

Previous studies have shown that use of Copper/Zinc SOD plasmid liposomes (an enzyme targeted to the cytoplasm) failed to produce significant radioprotection of the esophagus (12, 26). In these prior studies, targeting of the CU/ZnSOD to the mitochondrial membrane by attachment of the mitochondrial localization leader sequence of MnSOD to the transgene for CU/ZnSOD restored radioprotection to levels observed with MnSOD (12). Deleting the mitochondrial targeting sequence from the MnSOD transgene removed its radioprotective capacity. Previous studies established that ionizing irradiation damage protection by MnSOD-PL gene therapy is mediated at the level of the mitochondrial membrane, since targeting of enzyme action to that site was protective (12, 26, 50-52). Reduction of lipid peroxidation specifically in mitochondria of the esophageal cells *in situ* has not yet been demonstrated, but irradiation of MnSOD-PL-treated esophageal cells in culture reduced irradiation-induced apoptosis (38). Several published studies (45-49) suggest that there are tumor-specific lipid peroxidative changes and that redox balances that may differ between tumors and normal tissues. The present data further argue for the safe use of radioprotective MnSOD-PL gene therapy of the esophagus in lung cancer patients. The present report advocates for the use of MnSOD-PL gene therapy in clinical esophageal radiation protection (43, 44), and suggests that the mechanism of protection is in part attributable to gene therapy mediated reduction in irradiation-induced lipid peroxidation.

Acknowledgements

This research was supported by National Institutes of Health Grant no. R01-CA-83876, U.S.A.

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Received July 29, 2005
Accepted August 31, 2005