

Protection of Esophageal Multi-lineage Progenitors of Squamous Epithelium (Stem Cells) from Ionizing Irradiation by Manganese Superoxide Dismutase-Plasmid/Liposome (MnSOD-PL) Gene Therapy

YUNYUN NIU, HONGMEI SHEN, MICHAEL EPPERLY, XICHEN ZHANG,
SUHUA NIE, SHAONAN CAO and JOEL S. GREENBERGER

*Department of Radiation Oncology, University of Pittsburgh Cancer Institute,
200 Lothrop Street, Pittsburgh, PA 15213, U.S.A.*

Abstract. *Background: Intraesophageal manganese superoxide dismutase plasmid liposome (MnSOD-PL) gene therapy protects against irradiation damage. Materials and Methods: To determine whether esophageal side population (SP) stem cells were protected, epitope-tagged (hemagglutinin) (HA) MnSOD-PL was administered to C57BL/6J mice 24 hours prior to 30 Gy esophageal irradiation. SP cells were isolated, and apoptosis and multi-lineage vimentin/endothelin/F4/80 (macrophage) colonies in vitro were quantitated. Results: The number and percent of SP cells, apoptotic cells, or numbers of multi-lineage vimentin/endothelin/F4/80-positive in vitro colonies isolated from non-irradiated HA-MnSOD-PL-treated or 30 Gy-irradiated esophagus did not differ between groups. Irradiation in vitro significantly increased apoptosis in explanted non-SP cells from control ($p=0.021$) compared to MnSOD-PL-treated mice. Irradiation-induced cell division was significantly increased in SP cells from control-irradiated mice ($p=0.001$), but not MnSOD-PL-treated mice. Irradiation-induced apoptosis detected in vivo at 5 days was decreased by MnSOD-PL. Conclusion: MnSOD-PL gene therapy protects esophageal SP cells from irradiation in vitro and in vivo.*

A significant complication of chemoradiotherapy of non-small cell carcinoma of the lung is irradiation esophagitis (12, 13). Radiation esophagitis has been shown to be dependent upon total irradiation dose, radiation fraction

size, and volume of esophagus in the target volume (12, 13, 38). Systemic small molecule radioprotector agents have had little effect on ameliorating chemoradiotherapy-induced esophagitis (16, 17, 39). Effective chemotherapy drugs, including carboplatin and taxol, increase the severity of irradiation esophagitis, both in the degree of symptomatology (including dysphagia, dehydration and weight loss), and in limiting attempts at radiation dose escalation (12, 13). Furthermore, a principal hindrance to the inclusion of a third drug (Triplet) to effective dual agent chemotherapy has been irradiation esophagitis (14, 15).

We have previously demonstrated that intraesophageal administration of manganese superoxide dismutase-plasmid/liposome (MnSOD-PL) significantly reduces the severity of irradiation esophagitis in single fraction and fractionated irradiation protocols, and improves survival (1-5). In histopathological sections from irradiated mice, apoptotic cells have been identified in the basal squamous layers of the esophageal epithelium at 11-12 days after single fraction irradiation (1), a time coincident with the appearance of microulceration and significant weight loss and dehydration (1, 4, 5). In other studies, a population of esophageal stem cells was isolated by either a 7-day serial *in vitro* preplate technique or side population sorting of single cell suspensions (6, 7). These esophageal multilineage stem cells have been shown to have capacity for squamous, endothelial and macrophage-like cell differentiation *in vitro* based upon histochemical and morphological characteristics (7), and transplant by intravenous injection to the irradiated esophagus of recipient mice (6).

In the present studies, we sought to determine whether administration of an epitope-tagged (hemagglutinin) (HA) MnSOD-PL (11) reached the population of esophageal stem cells following intraoral administration, and whether protection from ionizing irradiation was in part mediated by

Correspondence to: Joel S. Greenberger, MD, Professor and Chairman, Department of Radiation Oncology, University of Pittsburgh Cancer Institute, 200 Lothrop Street, Pittsburgh, PA 15213, U.S.A. Tel: 412-647-3602, Fax: 412-647-6029

Key Words: Esophageal stem cell, ionizing irradiation, manganese superoxide dismutase-plasmid/liposome, gene therapy.

preservation of the numbers, viability and differentiation capacity of these side population (SP) cells. The results demonstrate that radioprotective MnSOD-PL gene therapy acts in part through protection of the SP cell population of these esophageal stem cells.

Materials and Methods

Mice, plasmid/liposomes and irradiation. Clinical grade MnSOD-PL was produced by Valentis, Inc. (Burlingame, CA, USA) under GMP conditions. Female C57BL/6NHsd (Harlan Sprague Dawley, Indianapolis, IN, USA) mice were injected intraesophageally with 100 µl of water by inserting a mouse feeding tube connected to a 1-cc syringe to the top of the esophagus where it was swallowed by the mice. This was followed with 100 µl of either MnSOD-PL or a control plasmid, pNGVL3-PL, containing 200 µg plasmid DNA. Groups of 5-10 female C57BL/6NHsd mice were injected with either water only or MnSOD-PL. In some of the experiments the mice were irradiated 24 h later to 30 Gy to the pulmonary cavity using a Varian 6 MeV linear accelerator at 200 cGy/min. The mice were shielded, as published (1), so that only the pulmonary cavity was irradiated. The abdomen, head and extremities remained outside the field of irradiation. The mice were sacrificed 48 h after irradiation and the SP cells were isolated and analyzed for changes in SP or non-SP numbers and apoptosis. In other experiments, the mice were injected with MnSOD-PL, pNGVL3-PL (a control plasmid) or water only, and sacrificed 24 h later with the SP and non-SP cells isolated and irradiated to 10 Gy. The cells were plated in 12-well tissue culture plates in DMEM median containing 10% fetal calf serum, penicillin/streptomycin and L-glutamine and the percent apoptotic cells was determined 24 h later.

Isolation of esophageal progenitor cells. Esophageal progenitor cells were isolated according to the published methods of sorting the SP cells from adult mouse esophagus (6). The esophagus of male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) was excised after the mice were dipped in 20% Betadine. Single cell suspensions were obtained by teasing the tissue in a solution containing 0.2% type XI collagenase, dispase (grade II, 240 U) and 0.1% trypsin and incubating for 1 h at 37°C. The cell suspensions in 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 Hoechst 33342 dye and the SP was identified by flow cytometry, as described previously (6). Briefly, the esophageal cells were resuspended at 10⁶ cells/ml in prewarmed Hank's Balanced Salt Solution (HBSS) with 2% fetal calf serum and 10 mM HEPES buffer, and Hoechst stain was added to a final concentration of 5 µg/ml. The cells were placed in a 37°C water bath for 90 min. The cells were washed and resuspended in cold HBSS, 2% FCS and 10 mM Hepes buffer for anti-CD45-FITC and anti-Ter 119-PC-7 staining to remove contaminating hematopoietic cells. Then 2 µg/ml propidium iodide was added for discrimination of dead cells. As a control preparation, the SP was purified from single live CD45-negative cells using a MoFlo high-speed cell sorter (DakoCytomation, Fort Collins, CO, USA). To quantify the SP cells, the cells were stained with anti-Ter119-PE-Cy7 and anti-CN45-FITC to

distinguish red blood cells. The frequency of SP cells was calculated by excluding the dead cells (PI+), hematopoietic (CD45+, Ter119+), debris and doublets (Figure 1). To confirm that the correct cells were identified, the population from cell sorting was blocked with 50 µM of Verapamil (6).

Demonstration of HA-SOD2 expression in the esophagus SP cells and in situ. To visualize and quantify the degree of manganese superoxide dismutase/MnSOD (SOD2) expression in the esophageal SP cells, mice were injected as described above with a hemagglutinin (HA) epitope tagged SOD2-PL or LacZ-PL (200 µg plasmid DNA plus 11 µl lipofectant in a final volume of 100 µl) (6). The mice were sacrificed 24 h later and the esophagus was excised and the SP cells isolated as described above. The SP cells were attached to a microscope slide by cyto centrifugation at 500 x g for 5 min and stained for HA expression as described previously (11). Briefly, for detection of epitope-tagged HA-SOD2, the cells were fixed in methanol for 10 min and washed three times in phosphate-buffered saline (PBS) for 10 min. The cells were covered with a primary antibody, rabbit mouse anti-hemagglutinin (Roche, Indianapolis, IN, USA), for 1 h in a humidified chamber and washed three times in PBS. The cells were covered with fluorescein isothiocyanate (FITC) anti-rabbit antibody (Calbiochem, La Jolla, CA, USA) for 1 h in the same chamber, washed three times for 10 min, dried and covered with Gelmount aqueous mounting medium anti-fading agents (Biomedica Corp., Foster City, CA, USA) (18) and observed under a fluorescent microscope.

Annexin V staining. Early apoptosis was determined by staining the cells with Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, Mountain View, CA, USA). The cells were washed twice in PBS, resuspended in 1x binding buffer at a density of 1x10⁶ cells/ml, 100 µl of cells transferred to a test tube and 5 µl of Annexin V-PE and 5 µl of 7-AAD were added. The cells were vortexed and incubated for 15 min at room temperature in the dark. To each tube, 400 µl of 1 x binding buffer was added and analyzed by flow cytometry within 1 h. The CyAn LX cytometer (DakoCytomation) was used for data acquisition.

Detection of irradiation-induced cell division. Control mice, as well as mice administered MnSOD-PL 24 h earlier, were irradiated to 30 Gy to the esophagus and sacrificed 24 h later. The esophagus was removed and stained for isolation of SP and non-SP cells. The cells were then incubated for 30 min with a FITC-conjugated anti-PCNA antibody. The percent of PCNA-positive SP and non-SP was calculated.

Multi-lineage epithelial colony assays. Esophageal SP cells were plated in methylcellulose-containing medium used in the assay of hematopoietic progenitor cells (7). Control cells were hematopoietic cells obtained from non-adherent cells harvested from >4-week-old murine long-term bone marrow cultures (LTBMcs), according to published methods (8). Cells were plated in 1% methylcellulose in Iscove's MDM media containing 30% FBS, 10% BSA, 50 ng/ml SCF, 10 mg/ml GM-SCF, IL-3, IL-6 and 3 U/ml erythropoietin (Pepro Tech, Inc., Rocky Hill, NJ, USA). Depending on the number of cells available from flow cytometric sorting, the number of cells plated ranged from 1x10⁴ to 4x10⁴ cells/plate. Cultures were maintained at 37°C with 5% CO₂ and >95% humidity (7).

The adherent cell colonies which had fallen to the plate surface were fixed in 1% glutaraldehyde. The non-adherent cells were stained with antibodies against vimentin, endothelin, or F4/80 by fixing the cells in methanol, washing twice in PBS, incubating for 2 h at room temperature in the presence of 1:100 dilutions with F4/80 (L11209, Caltag Laboratories, Burlingame, CA, USA), anti-mouse endothelin (Ab-2) (PC266, Calbiochem, San Diego, CA, USA), or anti-mouse vimentin antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The plates were scored for percent positive for the differentiation markers (vimentin, endothelin, F4/80). Positive controls were lung sections from irradiated mice, which were known positive for vimentin, endothelin and F4/80 (7). Negative controls were cells from the 32D cl 3 murine hematopoietic progenitor cell line (7).

Statistical analysis. A Student's *t*-test was used to compare changes in body weight when comparing mice injected with liposomes only, or the LacZ-PL complex to mice injected with the MnSOD-PL complex after irradiation.

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

HA-MnSOD reaches both SP and non-SP cells in intra-orally treated mouse esophagus. We first administered HA-MnSOD-PL to C57BL/6NHsd mice and 24 h later excised esophagus specimens and sorted esophageal SP cells. The esophagus was excised and digested into a single cell suspension and stained with Hoechst dye, anti-CD45-FITC and anti-Ter 119-PE-Cy7 to remove contaminating hematopoietic cells. The cells were sorted by flow cytometry into SP cells and non-SP cells (R5 and R6, respectively, in Figure 1). The cells were then stained with an anti-HA antibody. Mice receiving HA-MnSOD-PL showed both HA-positive SP and non-SP cells (Figure 2).

Overexpression of MnSOD has no effect on SP number following irradiation. To determine whether increased expression of MnSOD altered the number of SP cells following irradiation, mice were injected intraesophageally with water, pNGVL3-PL or MnSOD-PL, irradiated to 30 Gy to the esophagus and the number of SP cells analyzed 24 or 48 h later by Hoechst staining and flow cytometry. There was no change in the percent of SP cells (A) or in the total number (B) of SP cells per mouse following either intraesophageal MnSOD administration or irradiation (Figure 3).

MnSOD-PL treatment decreases irradiation-induced early apoptosis in SP and non-SP cells. Irradiation induces detectable (early) apoptosis 24 to 48 h after irradiation in cells within most tissues (30, 36). To determine whether increased expression of MnSOD protected esophageal SP or non-SP cells from irradiation-induced apoptosis, C57BL/6NHsd mice were injected intraesophageally with water (control), pNGVL3-PL or MnSOD-PL and were irradiated to 30 Gy 24 h later. The esophagus was excised 24, or 48 h after irradiation, digested into a single cell suspension, stained with Hoechst dye and Annexin V and analyzed by flow cytometry for the percent of SP and non-SP cells undergoing apoptosis. Following irradiation, there was no significant change in the percent of apoptotic SP or non-SP cells at 24 or 48 h (Figure 4A and 4B, respectively) in the esophagus from any of the groups.

We next irradiated isolated esophageal SP and non-SP cells explanted *in vitro* 24 h after the *in vivo* administration of water or MnSOD-PL. The cells were irradiated to 10 Gy, placed in DMEM medium, incubated at 37°C in a CO₂ incubator, stained for Annexin V 24 h after irradiation and analyzed by flow cytometry for the percent of cells undergoing apoptosis. The SP cells were relatively resistant to irradiation and demonstrated no significant change in percent apoptosis (8-10%) following irradiation *in vitro* (Figure 5A). Increased expression of MnSOD resulted in no significant change in the percent of apoptotic SP cells. In contrast, there was a significant increase ($p=0.021$, Figure 5B) in the percent of apoptosis following *in vitro* irradiation of esophageal non-SP cells. The increased expression of MnSOD by PL administration *in vivo* protected the non-SP cells from irradiation-induced apoptosis by the assay of *in vitro* explanted cells (Figure 5B).

MnSOD-PL treatment decreases irradiation-induced division of esophageal SP cells. Irradiation results in increased cell division of stem cells thought to be associated with repopulation responses to tissue cellular damage (31). One indication that MnSOD-PL treatment protects esophageal stem cells from irradiation would be inhibition of SP cell division. To determine whether overexpression of MnSOD slowed or prevented cell division following irradiation, mice were injected intraesophageally with water only, pNGVL3-PL, or MnSOD-PL, then irradiated 24 h later to 30 Gy to the esophagus. The mice were sacrificed 24 h after irradiation. The esophagus was removed, digested into a single cell suspension, stained with Hoechst dye and antibodies to PCNA (proliferating cell nuclear antigen), and analyzed by flow cytometry for the percent of SP and non-SP cells positive for PCNA as an indicator of cells undergoing cell division. Following irradiation there was a significant increase in the percent of PCNA-positive (+) SP cells in the control (water) or pNGVL3-PL-treated mice ($p<0.0001$ or $p=0.0014$,

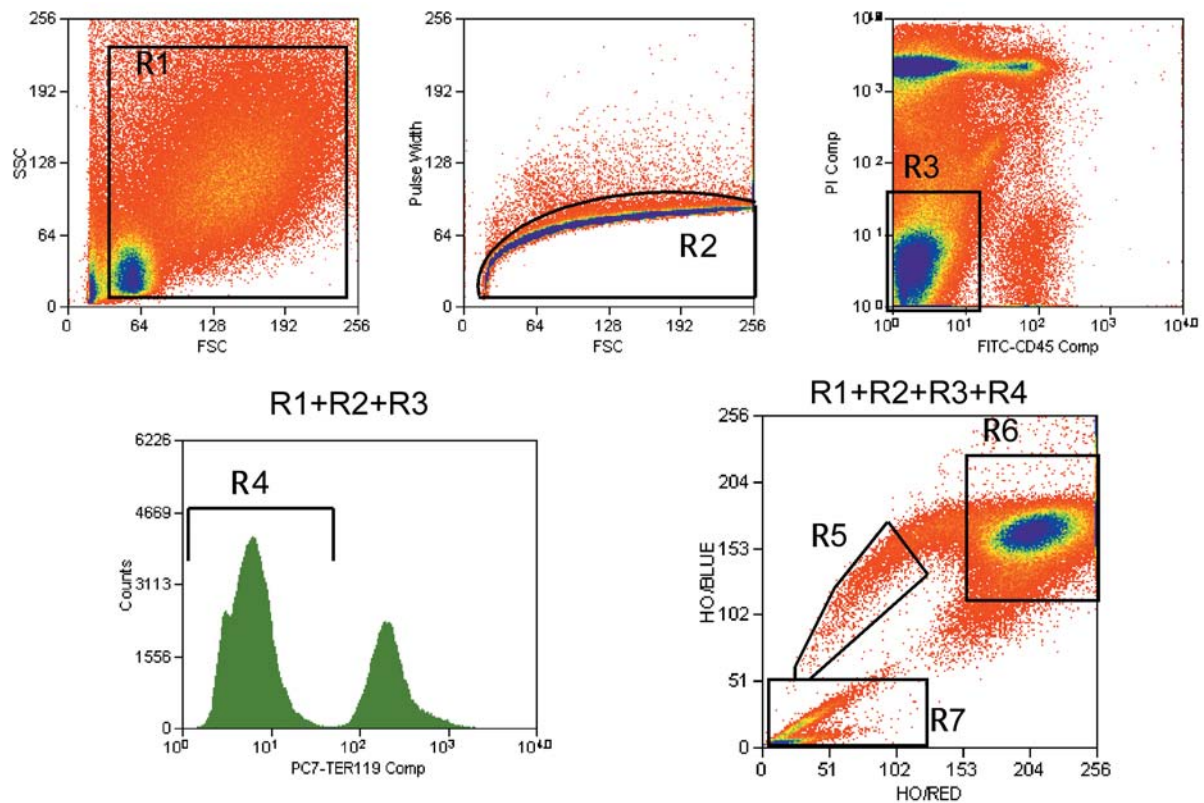


Figure 1. Isolation of SP and non-SP cells from the murine esophagus. C57BL/6NHsd mice were sacrificed and the esophagus harvested and digested into a single cell suspension. The cells were loaded with Hoechst33342 and stained with anti-CD45-FITC and anti-Ter119-PC-7. Propidium iodide was added to discriminate between live and dead cells. A MoFlo high-speed cell sorter (DakoCytomation) was used to acquire the SP and non-SP populations. To calculate the esophageal SP (R5), we excluded dead cells (PI+), hematopoietic cells (CD45+ and Ter119+) and debris (R7) and the doublets (R2).

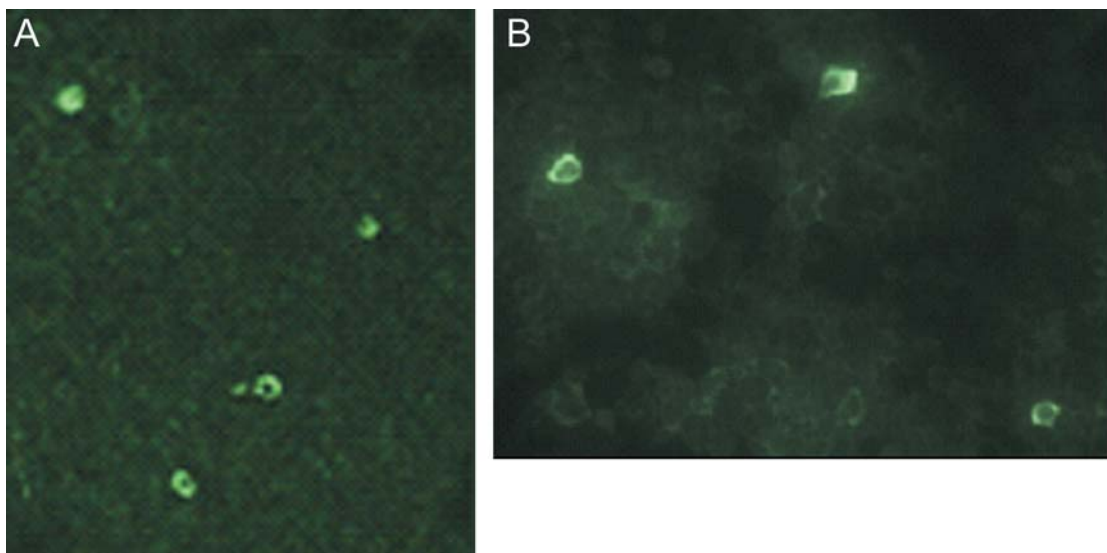


Figure 2. Expression of HA-MnSOD in both SP and non-SP cells following intraesophageally-administered HA-MnSOD-PL. C57BL/6NHsd mice received intraesophageally either water or HA-MnSOD-PL in a constant volume and were sacrificed 24 h later. The esophagus was removed and digested into a single cell suspension, stained with Hoescht dye, and sorted into SP and non-SP cells. The cells were cytopun onto microscope slides and stained with an anti-HA antibody. HA-positive cells were detected in both the SP (2A) and non-SP populations (2B).

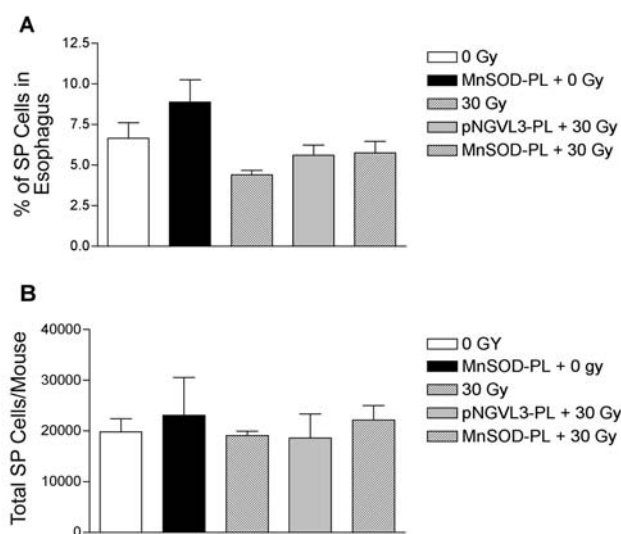


Figure 3. Lack of alteration of the percent of esophageal SP cells by 30 Gy irradiation. Mice were administered intraesophageally water, pNGVL3-PL or MnSOD-PL and irradiated 24 h later to the esophagus. The esophagus was removed 48 h later and digested into a single cell suspension and SP and non-SP cells sorted as described above. Following 30 Gy, there was no change in the percent of SP cells per esophagus (3A) or the total number of SP cells per mouse (3B). Administration of MnSOD resulted in no significant alteration in the number of SP cells.

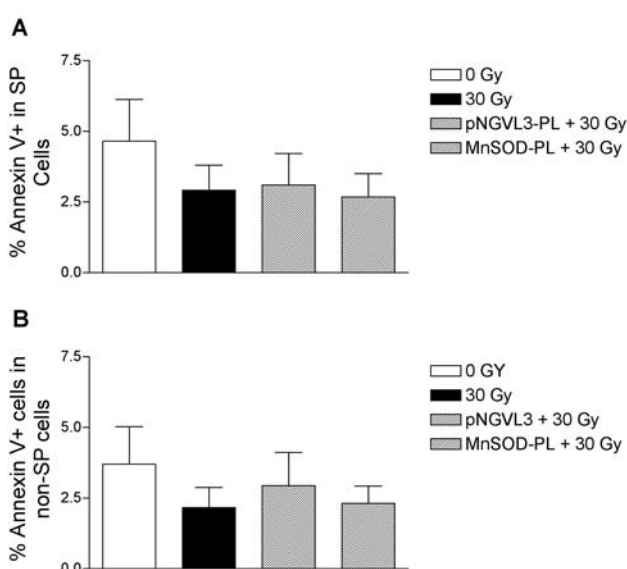


Figure 4. Apoptosis of in vivo irradiated esophageal cells detected in vitro following explant. C57BL/6NHsd female mice were administered water or MnSOD-PL and irradiated 24 h later to 30 Gy to the esophagus. The esophagus was collected 24 or 48 h later, digested into a single cell suspension and stained with Hoescht dye and Annexin V. The cells were then analyzed by flow cytometry for the percent apoptotic cells. There was no significant increase in the percent apoptotic cells following irradiation.

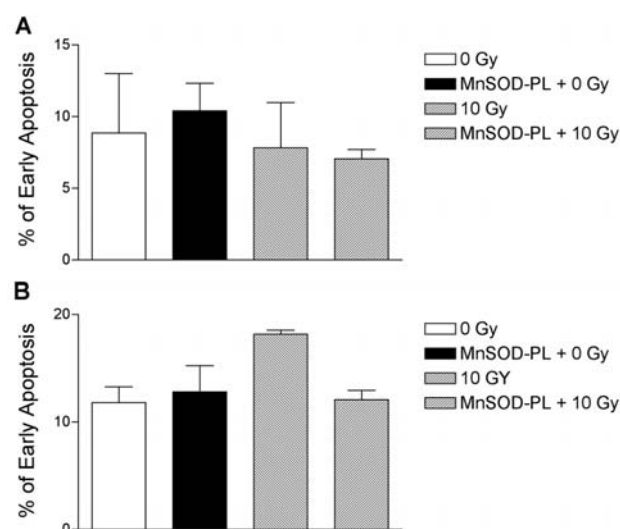


Figure 5. Administration of MnSOD-PL protects non-SP cells from irradiation-induced apoptosis. Mice were injected intraesophageally with water or MnSOD-PL and sacrificed 24 h later. The esophagus was removed, SP and non-SP cells isolated, irradiated at 0 or 10 Gy and plated in tissue culture media overnight. The cells were then stained for apoptosis using Annexin V and loaded with propidium iodide. The cells were sorted by flow cytometry and the percent of apoptotic cells determined. There was no significant change in the percent of SP apoptotic cells following irradiation in mice treated either with water alone or MnSOD-PL (Figure 5A). There was an increase in the percent of non-SP cells following irradiation ($p=0.021$) which was reduced by administration of MnSOD-PL 24 h before irradiation (Figure 5B).

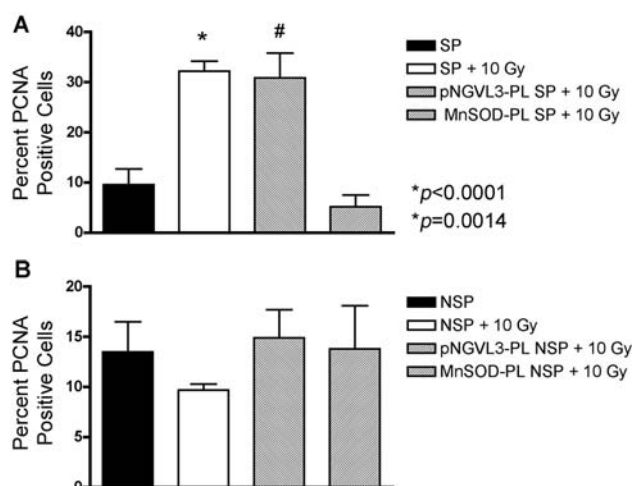


Figure 6. MnSOD inhibits irradiation-induced SP cell division. To determine if overexpression of MnSOD prevents irradiation-induced SP cell division, mice were injected with MnSOD-PL, pNGVL3-PL or water and irradiated to 30 Gy to the esophagus 24 h later. At 24 h after irradiation, the mice were sacrificed and the esophagus was excised, digested into single cell suspension, stained with Hoescht dye and antibody to PCNA. The cells were sorted into SP and non-SP populations, and the percent of dividing cells (PCNA+) was calculated. Following irradiation, there was an increase in the percent of dividing SP cells (PCNA+), control-irradiated and pNGVL3-PL-treated cells which were inhibited by overexpression of MnSOD (Figure 6A). Non-SP cells demonstrated no significant changes in cells undergoing cell division (Figure 6B).

Figure 6A). In contrast, mice treated with MnSOD-PL demonstrated no increased cell division in the SP cell population (Figure 6A). There was no significant increase in cell division independent of MnSOD-PL treatment in the irradiated non-SP cell population (Figure 6B). Thus, most cell division following irradiation occurred in the SP (stem cell) subset. The data are in support of the hypothesis that primitive SP cells divide and differentiate into the non-SP population, including those squamous cells of the esophagus, to facilitate repair of irradiation-induced damage.

MnSOD-PL treatment results in decreased late apoptosis in the esophagus in vivo. In the esophagus of control mice, irradiation-induced apoptosis was detected 3 to 5 days after irradiation and peaked at about 7 days (Figure 7). In mice treated with MnSOD-PL prior to irradiation, there was reduced apoptosis at all time points-tested compared to the control-irradiated mice (Figure 7).

HA-MnSOD-PL administration protects the in vitro multi-lineage differentiation capacity of cells found in the SP population. We next determined whether the esophageal SP cells with differentiation capacity scored *in vitro* according to published methods (7) were significantly altered by HA-MnSOD-PL intraesophageal gene therapy. Mice in each group, control, irradiated, HA-MnSOD-treated, and HA-MnSOD-treated, then irradiated mice were sacrificed and esophageal SP cells isolated. Esophageal SP cells were plated 100 cells per well in six-well plates according to published methods (7). Flattened colonies on the plate's surface were enumerated at day 7, then stained by histochemical techniques for vimentin (squamous), endothelin (endothelial cell specific) and F480 (macrophage cell specific). The results are shown in Table I.

There was no significant change in the differentiation capacity of the SP or non-SP cells in pNGVL3-PL- or MnSOD-PL-treated populations, or in those that were from irradiated mice compared to non-irradiated mice. Non-adherent colonies were not detected and only adherent-colonies were detectably formed in each cell group tested. The colonies from each group showed similar differentiation patterns, except for SP cells from the control-irradiated and pN6VL3-PL irradiated mice, which showed a decrease in cells with positive expression of F4/80 ($p=0.0472$ and 0.0464 , respectively). These results confirm and extend our previous publication, demonstrating that SP cell sorting of esophageal stem cell subsets results in isolation of a population of cells that differentiate *in vitro* into colonies containing multi-lineages (7).

Discussion

Organ-specific radioprotective gene therapy utilizing MnSOD-PL administration has been shown to be effective in protecting the murine lung (18-21), oral cavity/oropharynx

(11), bladder (22), intestine (27) and esophagus (1-3). In studies of single fraction and fractionated irradiation, single administration or multiple administration of MnSOD-PL by intra-oral injection and induced swallowing in non-anesthetized mice has been shown to have a significant radioprotective effect on the esophagus and oral cavity (1-3, 11). In the present studies, we sought to determine whether the mechanism of radiation protection was in part attributable to improved survival of esophageal stem cells with capacity for squamous, endothelial and macrophage differentiation (7).

Mice received an epitope-tagged MnSOD-PL complex 24 h prior to irradiation. The results showed that HA-MnSOD reached esophageal stem cells that were sorted in the SP fraction of single cell suspensions of esophagus removed from mice. The administration of MnSOD-PL resulted in a significant fraction of SP cells being tagged with detectable HA-MnSOD transgene product. These results confirm our previous studies, using immunofluorescence detection of HA-MnSOD in tissue sections, that epitope-tagged transgene reaches basal layers within the esophagus (5, 11).

Following irradiation there was no change in the number of esophageal SP cells per mouse, or the percent of SP cells within the total population of esophageal cells between control and MnSOD-PL-treated mice. In most tissues, apoptosis occurs within 1 to 2 days following irradiation (2), but in the esophagus no apoptosis was detectable in the first 3 days in the control or MnSOD-PL-treated mice (Figure 4). The *in vitro* irradiation of explanted isolated SP or non-SP cells from the esophagus of control or MnSOD-PL-treated mice did reveal increased apoptosis in the control-irradiated non-SP cells, but not in the non-SP cells from MnSOD-PL-treated mice (Figure 5). There was no irradiation-induced increase in apoptosis in SP cells from either control or MnSOD-PL-treated mice. The data are in agreement with prior publications, demonstrating that bone marrow stem cells found in the SP subset are more resistant to irradiation-induced apoptosis (24, 29).

While there was no detectable increase in the number of esophageal SP cells following irradiation, there was a detectable increase in the number of SP cells dividing in control-irradiated or control plasmid/liposome pNGVL3-treated mice (Figure 6). MnSOD-PL pre-treatment blocked irradiation-induced cell division in these esophageal SP cells. The data indicate that irradiation-induced damage to the esophagus promotes cell division and results in differentiation of esophageal SP cells. If cell division is a damage response of esophageal SP cells perhaps attributable to changes in the esophageal microenvironment, then prevention of cell division by MnSOD-PL may have acted at the level of the esophageal stromal cells. Further studies are being carried out to test this hypothesis.

Apoptosis *in vivo* following irradiation of the esophagus significantly increased around day 7 after 30 Gy. Irradiation-induced apoptosis *in vitro* is a result of DNA strand breaks leading to translocation of BAX and SAP kinases to the mitochondria, decreased mitochondrial potential, release of cytochrome C into the cytoplasm and initiation of apoptosis (2). These subcellular events occur in the first 24 to 48 h after irradiation, while the "late" apoptosis *in vivo* began at days 5 to 7. These observations may indicate that the apoptotic stimulus at days 5-7 is indirect through irradiated stromal cell responses (perhaps cytokine release). We have previously demonstrated that there are increased levels of IFN- γ at days 4 and 7 following irradiation and increases in TNF- α at day 7 (5). Administration of MnSOD-PL before irradiation prevented increases in these cytokines (5). Since TNF- α and Inf- γ have been shown to induce apoptosis (5-7), these cytokines may be responsible for the apoptosis detected around days 5-7.

HA-MnSOD administration itself did not induce a shift of cells from the esophageal non-SP to the SP fraction of cell sorting, nor did it improve the survival of explanted cells. The technique for excision of the esophagus and preparation of single cell suspensions prior to SP cell sorting was developed specifically to optimize survival of explanted cells (6). Other studies have demonstrated that the serial 7-day preplate technique, in which non-adherent cells from each whole esophagus culture are transplanted into new culture dishes daily for 7 days, results in isolation of a population of non-adherent cells at day 7 that have some *in vivo* transplant properties similar to the freshly sorted SP cell population (6). In contrast to freshly sorted esophageal SP cells, day 7 preplate cells showed a propensity for multilineage differentiation capacity to vimentin/endothelin/ F480-positive cells (7). The data support the observation that 7-day *in vitro* culture induced differentiation of the remaining non-adherent cells compared to those in freshly sorted SP populations (7). Therefore, esophageal origin SP cells may change phenotype, as do other stem cell sorted subsets (41).

In the present studies, we sought to determine whether the administration of MnSOD-PL altered the fraction of SP cells with dual or trilineage differentiation capacity. The results of *in vitro* culture showed that the HA-MnSOD gene therapy did not push differentiation of SP cells to multiple lineages following explant to *in vitro* culture.

Self-renewing stem cell populations isolated from several epithelial organs have been shown to be critical for the repair of ionizing irradiation damage (23-25) and other forms of tissue injury (26-30, 44). The intestinal crypt assay of Withers *et al.* (25) first demonstrated the recolonization of irradiated intestine by stem cells located in the crypt areas. Several studies have documented the capacity of bone marrow-derived progenitors for epithelial cells to

reconstitute irradiated epithelial cell-containing tissues (27-29) including the esophagus (6). Thus, multiple lines of evidence suggest that stem cell populations are critical for both the tissue repopulation response to irradiation (24, 28, 29) and the maintenance of organ integrity during the post-radiation recovery period (30).

The use of stem cell transplants to repair damaged organs has been proposed. Previous attempts with esophageal stem cells or bone marrow stem cells to repair irradiation-induced damage to the esophagus have been performed 2 h after irradiation (6, 7). The present results demonstrate that detectable apoptosis does not occur until 5 to 7 days after irradiation *in vivo* and suggests that transplantation of esophageal stem cells may be more successful if performed at this later time-point. Apoptotic cell death may provide a niche in which engrafted or circulating stem cells from non-irradiated sites can home to a microenvironment which is more conducive for cell division repair of the irradiation-induced damage. The reason for this late esophageal apoptosis is unknown, but may be related to a bystander effect of irradiation on the underlying mucosa, or cytokine response from the irradiated esophageal squamous cells.

In other studies, the preservation by MnSOD-PL treatment of stem cells by reducing their irradiation-induced cycling has been shown (31). Several potential protective mechanisms of antioxidant MnSOD-PL therapy during *in vivo* radioprotection have been recently reviewed (32-37). The tissue damage effects of ionizing irradiation include cytokine-mediated bystander cell killing (40, 42, 43) and free radical-mediated bystander cell killing (30) effects, which are both ameliorated by MnSOD-PL gene therapy (36). The success of MnSOD-PL gene therapy in protecting self-renewing normal stem cells within the murine esophagus from irradiation esophagitis argues favorably for translation of this technique into clinical protocols for protection of the esophagus from chemoradiotherapy damage in non-small cell lung carcinoma patients.

Acknowledgements

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

References

- 1 Stickle RL, Epperly MW, Klein E, Bray JA and Greenberger JS: Prevention of irradiation-induced esophagitis by plasmid/liposome delivery of the human manganese superoxide dismutase (MnSOD) transgene. *Radiat Oncol Invest Clin Basic Res* 7(6): 204-217, 1999.
- 2 Epperly MW, Sikora C, Defilippi S, Gretton J, Zhan Q, Kufe DW and Greenberger JS: MnSOD inhibits irradiation-induced apoptosis by stabilization of the mitochondrial membrane against the effects of SAP kinases p38 and Jnk 1 translocation. *Radiat Res* 157: 568-577, 2002.

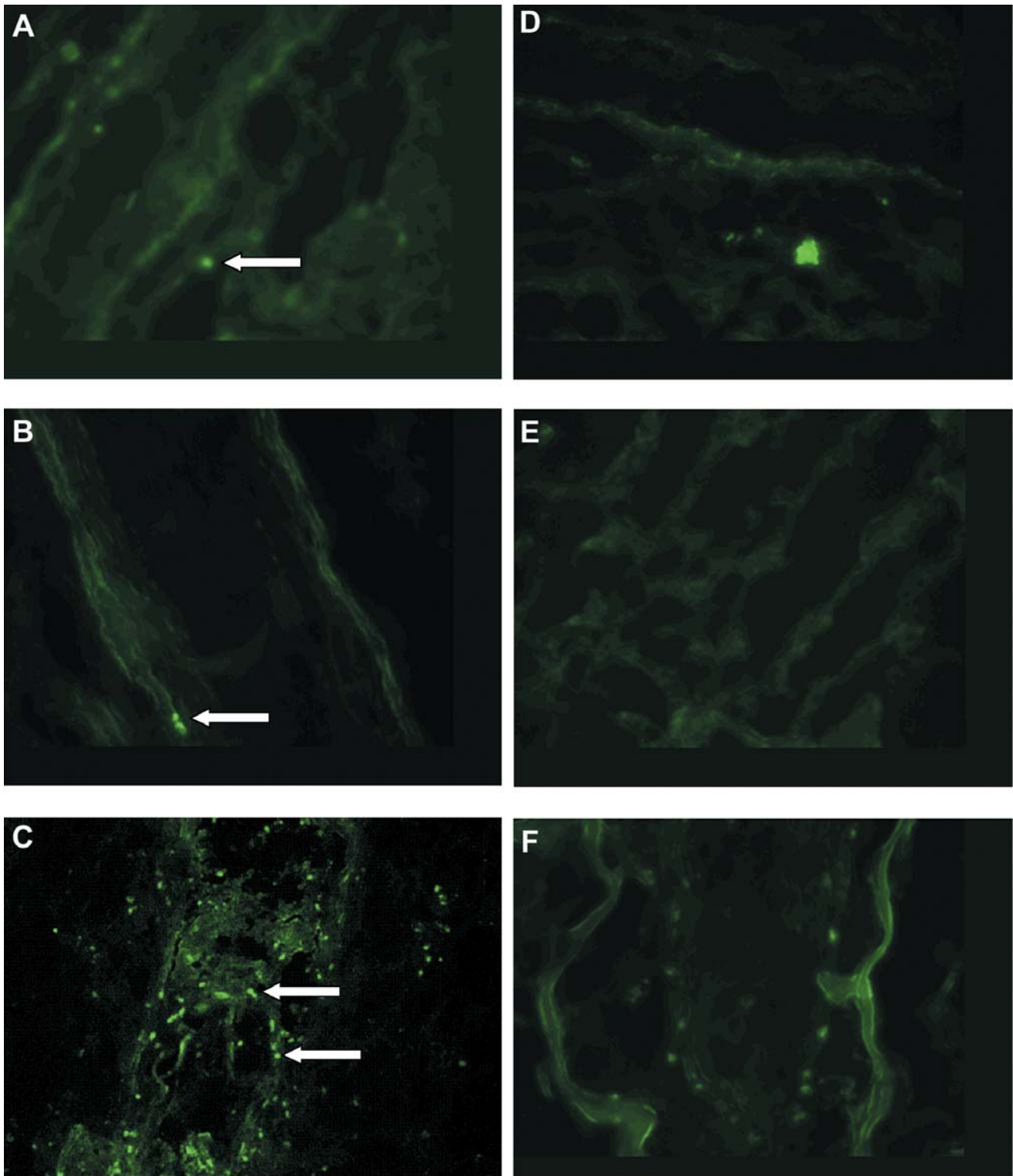


Figure 7. Inhibition of "Late" *in vivo* irradiation-induced apoptosis in the esophagus by MnSOD-PL administration. C57BL/6NHsd mice were injected intraesophageally with water or MnSOD-PL and irradiated to 30 Gy to the esophagus 24 h after injection. The mice were sacrificed 0, 1, 3, 5 or 7 days after irradiation. The esophagus was removed, frozen in OCT, sectioned, stained for apoptosis using a Tunel Assay (Promega), and observed under a fluorescent microscope. Little apoptosis was detected on day 1 (Figure 7A) and further on day 3 (Figure 7B), increasing significantly by day 7 in the control-irradiated mice (Figure 7C). Mice injected intraesophageally with MnSOD-PL demonstrated little apoptosis at day 1, day 3 (Figure 7D and 7E, respectively) and at day 7 (Figure 7F).

Table I. Differentiation capacity of murine esophageal SP compared to non-SP cells.

Group	SP			Non-SP		
	Endothelin	Vimentin	F4/80	Endothelin	Vimentin	F4/80
Control	45.5±8.1	39.4±10.0	26.8±2.1	38.5±14.9	48.2±7.2	25.8±8.8
Control + 30 Gy	45.8±5.4	33.8±6.1	18.0±3.1*	41.5±8.9	36.0±3.0	29.0±6.7
pNGVL3-PL + 30 Gy	53.1±7.8	36.0±5.1	11.5±6.5*	38.0±4.4	20.0±8.5	17.6±7.2
MnSOD-PL + 30 Gy	44.8±6.4	33.7±8.6	21.4±7.3	37.3±4.3	42.3±3.7	21.3±3.5

SP and non-SP cells were isolated from control, 30 Gy-irradiated, pNGVL3-PL-treated then 30 Gy-irradiated, or MnSOD-PL-treated then 30 Gy-irradiated mice. The cells were then grown in tissue culture for 7 days according to the Methods at which time they were analyzed for the expression of endothelin, vimentin and F4/80 (macrophage) markers using the immunohistochemistry method as published (7). The cells in each colony of >50 cells per colony were examined using a fluorescent microscope and the percent of cells expressing each marker was calculated based on scoring of at least 100 colonies from each of triplicate experiments. A significant decrease was detected in the expression of F4/80 in irradiated SP cells compared to control or MnSOD-PL-pretreated then irradiated mice ($p=0.0472$) (*).

- Epperly MW, Gretton JE, Bernarding M, Nie S, Rasul B and Greenberger JS: Mitochondrial localization of copper/zinc superoxide dismutase (Cu/ZnSOD) confers radioprotective functions *in vitro* and *in vivo*. *Radiat Res* 160: 568-578, 2003.
- Epperly MW, Kagan VE, Sikora CA, Gretton JE, Defilippi SJ, Bar-Sagi D and Greenberger JS: Manganese superoxide dismutase-plasmid/liposome (MnSOD-PL) administration protects mice from esophagitis associated with fractionated irradiation. *Int J Cancer (Radiat Oncol Invest)* 96(4): 221-233, 2001.
- Epperly MW, Gretton JA, Defilippi SJ, Sikora CA, Liggitt D, Koe G and Greenberger JS: Modulation of radiation-induced cytokine elevation associated with esophagitis and esophageal stricture by manganese superoxide dismutase-plasmid/liposome (SOD-PL) gene therapy. *Radiat Res* 155: 2-14, 2001.
- Epperly Michael W, Guo Hongliang, Shen Hongmei, Niu Yunyun, Zhang Xichen, Jefferson Mia, Sikora Christian A and Greenberger JS: Bone marrow origin of cells with capacity for homing and differentiation to esophageal squamous epithelium. *Radiat Res* 162: 233-240, 2004.
- Epperly MW, Shen H, Jefferson M and Greenberger JS: *In vitro* differentiation capacity of esophageal progenitor cells with capacity for homing and repopulation of the ionizing irradiation damaged esophagus. *In Vivo* 18: 675-685, 2004.
- Sakakeeny MA and Greenberger JS: Granulopoiesis longevity in continuous bone marrow cultures and factor dependent cell line generation: significant variation among 28 inbred mouse strains and outbred stocks. *J Nat Canc Inst* 68: 305-317, 1982.
- Greenberger JS, Anderson J, Berry LA, Epperly MW, Cronkite EP and Boggs SS: Effects of irradiation of CBA/Ca mice on hematopoietic stem cells and stromal cells in long-term bone marrow cultures. *Leukemia* 10(3): 514-527, 1996.
- Epperly MW, Sikora C, Defilippi S, Bray J, Koe G, Liggitt D, Luketich JD and Greenberger JS: Plasmid/liposome transfer of the human manganese superoxide dismutase (MnSOD) transgene prevents ionizing irradiation-induced apoptosis in human esophagus organ explant culture. *Int J Cancer (Radiation Oncol Invest)* 90(3): 128-137, 2000.
- Guo HL, Seixas-Silva JA, Epperly MW, Gretton JE, Shin DM, Bar-Sagi D, Archerand Herb and Greenberger JS: Prevention of irradiation-induced oral cavity mucositis by plasmid/liposome delivery of the human manganese superoxide dismutase (MnSOD) transgene. *Radiat Res* 159: 361-370, 2003.
- Saeko H, Kayoko T, Masahiro E, Yoshikazu K, Miyako S, Tetsuji K, Yoshio H, Kayoko O, Yoshiki T, Michio K and Mitsuyuki A: Dosimetric predictors of radiation esophagitis in patients treated for non-small-cell lung cancer with carboplatin/paclitaxel/radiotherapy. *IJROBP* 51(2): 291-295, 2001.
- Patel AB, Edelman MJ, Kwok Y, Krasna MJ and Suntharalingam M: Predictors of acute esophagitis in patients with non-small-cell lung carcinoma treated with concurrent chemotherapy and hyperfractionated radiotherapy followed by surgery. *IJROBP* 60(4): 1106-1112, 2004.
- Socinski MA, Rosenman JG, Schell MJ, Halle J, Russo S, Rivera P, Clark J, Limentani S, Fraser R, Mitchell W and Detterbeck FC: Induction Carboplatin/Paclitaxel followed by concurrent Carboplatin/Paclitaxel and dose-escalating conformal thoracic radiation therapy in unresectable stage IIIA/B nonsmall cell lung carcinoma. A modified phase I trial. *Cancer* 89: 534-542, 2000.
- Socinski MA, Rosenman JG, Halle J, Schell MJ, Lin Y, Russo S, Rivera MP, Clark J, Limentani S, Fraser R, Mitchell W and Detterbeck FC: Dose-escalating conformal thoracic radiation therapy with induction and concurrent Carboplatin/Paclitaxel in unresectable stage IIIA/B nonsmall cell lung carcinoma. A modified phase I/II trial. *Cancer* 92: 1213-1223, 2001.
- Boccia R, Rani AP, Bourhis J, Brizel D, Daly C, Holloway N, Hymes S, Koukourakis M, Kozloff M, Turner M and Wasserman T: Assessment and management of cutaneous reactions with amifostine administration: findings of the ethylol (Amifostine) cutaneous treatment advisory panel (ECTAP). *IJROBP* 60(1): 302-309, 2004.
- Komaki R, Lee JS, Milas L, Lee HK, Fossella FV, Herbst RS, Allen PK, Liao Z, Stevens CW, Lu C, Zinner RG, Papadimitrakopoulou VA, Kies MS, Blumenschein GR, Pisters

- KM, Glisson BS, Kurie J, Kaplan B, Garza VP, Mooring D, Tucker SL and Cox JD: Effects of amifostine on acute toxicity from concurrent chemotherapy and radiotherapy for inoperable non-small-cell lung cancer: report of a randomized comparative trial. *IJROBP* 58(5): 1369-1377, 2004.
- 18 Epperly MW, Defilippi S, Sikora C, Gretton J, Kalend K and Greenberger JS: Intratracheal injection of manganese superoxide dismutase (MnSOD) plasmid/liposomes protects normal lung but not orthotopic tumors from irradiation. *Gene Ther* 7(12): 1011-1018, 2000.
- 19 Epperly MW, Defilippi S, Sikora C, Gretton J and Greenberger JS: Radioprotection of lung and esophagus by overexpression of the human manganese superoxide dismutase transgene. *Mil Med* 167(1): 071, 2002.
- 20 Guo H, Epperly MW, Bernarding M, Nie S, Gretton J, Jefferson M and Greenberger JS: Manganese superoxide dismutase-plasmid/liposome (MnSOD-PL) intratracheal gene therapy reduction of irradiation-induced inflammatory cytokines does not protect orthotopic lewis lung carcinomas. *In Vivo* 17: 13-22, 2003.
- 21 Epperly MW, Sikora CA, Defilippi S, Gretton JE and Greenberger JS: Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. *AJRCMB* 29: 213-224, 2003.
- 22 Kanai A, Epperly MW, Pearce L, Birdier L, Zeidel M, Myers S, Greenberger J, deGroat W, Apodaca G and Peterson J: Differing roles of mitochondrial nitric oxide synthase in cardiomyocytes and urothelial cells. *Am J Physiol Heart Circ Physiol* 286: H13-H21, 2004.
- 23 Guo HL, Wolfe D, Epperly MW, Huang S, Liu K, Glorioso JC, Greenberger J and Blumberg D: Gene transfer of human manganese superoxide dismutase protects small intestine villi from radiation injury. *J Gastrointest Surg* 7: 229-236, 2003.
- 24 Herzog Erica L, Chai Li and Krause Diane S: Plasticity of marrow-derived stem cells. *Blood* 102(10): 3483-3490, 2003.
- 25 Withers HR, Mason K, Reid BO, Dubrasky N, Barkley HT, Brown BW and Smathers JB: Response of mouse intestine to neutrons and gamma rays in relation to dose fractionation and division cycle. *Cancer* 34: 39-47, 1974.
- 26 Prindull G and Zipori D: Environmental guidance of normal and tumor cell plasticity: epithelial mesenchymal transitions as a paradigm. *Blood* 103(8): 2892-2900, 2004.
- 27 Houghton JM, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR and Wang TC: Gastric cancer originating from bone marrow-derived cells. *Science* 306: 1568-1572, 2004.
- 28 Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S and Sharkis SJ: Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 105: 369-377, 2001.
- 29 Theise ND and Krause DS: Toward a new paradigm of cell plasticity. *Leukemia* 16: 542-548, 2002.
- 30 Mothersill C and Seymour CB: Radiation-induced bystander effects – implications for cancer. *Nature Rev Cancer* 4: 158-165, 2004.
- 31 Epperly MW, Carpenter M, Agarwal A, Mitra P, Nie S and Greenberger JS: Intra-oral manganese superoxide dismutase plasmid liposome radioprotective gene therapy decreases ionizing irradiation-induced murine mucosal cell cycling and apoptosis. *In Vivo* 18: 401-410, 2004.
- 32 Greenberger JS, Kagan VE, Pearce L, Boriseniao G, Tyurina Y and Epperly MW: Modulation of redox signal transduction pathways in the treatment of cancer. *Antiox Redox Signal* 3(3): 347-359, 2001.
- 33 Pearce LL, Epperly MW, Greenberger JS, Pitt B and Peterson J: Identification of respiratory complexes I and III as mitochondrial sites of damage following exposure to ionizing radiation and nitric oxide. *Nitric Oxide: Biol and Chem* 5(2): 128-136, 2001.
- 34 Epperly MW, Bernarding M, Gretton J, Jefferson M, Nie S and Greenberger JS: Overexpression of the transgene for manganese superoxide dismutase (MnSOD) in 32D cl 3 cells prevents apoptosis induction by TNF- α , IL-3 withdrawal and ionizing irradiation. *Exp Hematol* 31(6): 465-474, 2003.
- 35 Epperly MW, Osipov AN, Martin I, Kawai K, Borisenko GG, Jefferson M, Bernarding M, Greenberger JS and Kagan VE: Ascorbate as a "redox-sensor" and protector against irradiation-induced oxidative stress in 32D cl 3 hematopoietic cells and subclones overexpressing human manganese superoxide dismutase. *IJROBP* 58(3): 851-861, 2004.
- 36 Greenberger JS and Epperly MW: Radioprotective antioxidant gene therapy: potential mechanisms of action. *Gene Ther Molec Biol (GTMB)* 8: 31-44, 2004.
- 37 Perry Y, Epperly MW, Fernando HC, Klein E, Finkelstein S, Greenberger JS and Luketich JD: Photodynamic therapy induced esophageal-stricture – an animal model: from mouse to pig. *J Surg Res* 123: 67-74, 2005.
- 38 Ahn S-J, Kahn D, Zhou S, Yu X, Hollis D, Shafman TD and Marks LB: Dosimetric and clinical predictors for radiation-induced esophageal injury. *IJROBP* 61(2): 335-347, 2005.
- 39 Movasa B, Scott C, Langer C, Werner-Wasik M, Nicolaou N and Komaki R: Randomized trial of Amifostine in locally advanced non-small-cell lung cancer patients receiving chemotherapy and hyperfractionated radiation: Radiation Therapy Oncology Group Trial 98-01. *J Clin Oncol* 23: 2145-2154, 2005.
- 40 Rube CE, Uthe D, Wilfert F, Ludwig D, Yang K, Konig J, Palm J, Schuck A, Willich N, Remberger K and Rube C: The bronchiolar epithelium as a prominent source of pro-inflammatory cytokines after lung irradiation. *IJROBP* 61(5): 1482-1492, 2005.
- 41 Zhang C and Lodish HF: Murine hematopoietic stem cells change their surface phenotype during *ex vivo* expansion. *Blood* 105(11): 4314-4320, 2005.
- 42 Kamata H, Honda S, Maeda S, Chang L, Hirata H and Karin M: Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120: 649-661, 2005.
- 43 Yoon Y-S, Lee J-H, Hwang S-C, Choi KS and Yoon G: TGF β 1 induces prolonged mitochondrial ROS generation through decreased complex IV activity with senescent arrest in Mv1Lu cells. *Oncogene* 24: 1895-1903, 2005.
- 44 Kleeberger W, Versmold A, Rothamel T, Glockner S, Bredt M, Haverich A, Lehmann U and Kreipe H: Increased chimerism of bronchial and alveolar epithelium in human lung allografts undergoing chronic injury. *Am J Pathol* 162(5), 2003.

Received July 11, 2005

Accepted July 29, 2005