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Modulation of Total Body Irradiation Induced Life Shortening by Systemic Intravenous MnSOD-Plasmid Liposome Gene Therapy

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Abstract

To determine whether systemic administration of MnSOD-PL protected mice from the acute hematopoietic syndrome as well as delayed death following total body irradiation (TBI), C57BL/6J mice received intravenously 100 μ l liposomes containing 100 μ g of human MnSOD-transgene plasmid 24 hours prior to 9.5 Gy or 1.0 Gy. The dose of 9.5 Gy was lethal to 42% of irradiated control female and 74% of irradiated control male mice respectively at 30 days with bone marrow hypocellularity consistent with the hematopoietic syndrome. A statistically significant increase in survival was detected in MnSOD-PL treated compared to 9.5 Gy irradiated control female mice out to 400 days, and in male mice out to 340 days. The incidence of tumors was similar between surviving groups. Between 350 to 600 days, outcome was similar for both MnSOD-PL treated and control irradiated groups consistent with aging with no difference in gross or microscopic pathologic evidence of tumors. Male and female mice receiving 1.0 Gy TBI showed irradiation induced life shortening after 120 days that was decreased by MnSOD-PL administration, and was associated with no increase in rate of tumor associated death. Therefore, systemic MnSOD-PL radioprotective gene therapy is not associated with a detectably higher incidence of late carcinogenesis.

INTRODUCTION

The search for radiation protective and radiation damage mitigating agents to ameliorate the hematopoietic, and gastrointestinal syndromes associated with acute irradiation damage, has raised the question of whether agents used in such therapies will increase the likelihood of radiation leukemogenesis and carcinogenesis (4,13,15–16,18–19). In particular, radioprotective agents which decrease radiation induced apoptosis and/or elaboration of inflammatory cytokines have been suggested to be potentially deleterious due to the consequence of allowing radiation damaged cells to survive in self-renewing stem cell populations of the bone marrow and gastrointestinal tract (4,13–15). Such survival of damaged cells could theoretically lead to an increased rate and/or total incidence of new transformation events in those and related organs (16).

Antioxidant gene therapy studies have included the use of manganese superoxide dismutase-Plasmid Liposomes (MnSOD-PL). Overexpression of the mitochondrial localized MnSOD gene product has been shown to decrease ionizing radiation induced apoptosis of cells in culture (20–21). Organ specific application of MnSOD-PL reduces both acute and chronic radiation damage to the murine lung (22), esophagus (23), oral cavity (24), bladder (25), and intestine

(26). The potential of MnSOD-PL treatment to exacerbate the long-term life shortening effects of irradiation have not been reported.

In the present study, we determined the effects of systemic administration of MnSOD-PL given intravenously to C57BL/6J female and male mice, 24 hours prior to total body irradiation. Previous studies have demonstrated that the MnSOD transgene product is detectable in transfected tissues at 24 hrs (23) and transgene mRNA is detectably elevated for 24–96 hours after organ specific administration (27–28). The percent of cells transduced in organ specific radioprotective gene therapy has been shown to be 50–80% (28) with persistence of transgene in endothelial cells of the lung outlasting expression in alveolar type II cells or macrophages (28).

We considered the possibility that MnSOD-PL prophylaxis might protect/reduce the carcinogenic response to irradiation as has been suggested in prior publications using other antioxidants.(42)

In the present study, we determined the radioprotective effect of systemic MnSOD-PL gene therapy in total body irradiated mice with respect to the hematopoietic syndrome using the LD 50/30 dose of 9.5Gy. All surviving animals in groups receiving 9.5 Gy, or a lower dose of 1.0 Gy were followed for long term effects on survival from total body irradiation compared to surviving irradiated controls and age matched unirradiated mice. The effect of MnSOD-PL administration on altering long-term irradiation induced effects was measured. The results demonstrate improved survival of total body irradiated mice by MnSOD-PL radioprotective gene therapy with no associated deleterious late detectably increased induction of malignancy.

MATERIALS AND METHODS

Mice and Irradiation

C57BL/6J male and female mice 20–23 grams, 6–8 weeks old, in groups of 50 were housed 5 per cage, at our center using standard laboratory chow and deionized water according to Institutional IACUC protocols. Mice received total body irradiation delivered by Cesium 137 J.L. Shepherd Mark I, Model 68 irradiator at 80 cGy per minute, 24 hrs after intravenous administration of MnSOD-PL. For irradiation, five mice were placed in a “rig” which was a circular plexiglass pie plate (made by the University of Pittsburgh, School of Pharmacology Machine Shop) 21.5 cm in diameter and 7.5 cm in height. The mice were not immobilized but had freedom to walk around inside the plate. The plate was placed on a turntable which continuously turned at 1 rotation – per 8 seconds during irradiation insuring that all mice received the same radiation dose.

All mice were examined daily by a dedicated laboratory technician. Dead or dying mice were immediately removed, and necropsy carried out with examination of all organs and organ systems for evidence of malignancy. Histopathologic evaluation of bone marrow, gastrointestinal tract, and all mass lesions was carried out on formalin fixed, then Hematoxylin and Eosin stained histopathologic slides. Wrights-Giemsa stained bone marrow touch preparations were obtained from fresh femur marrow prior to fixation.

The control mice did not have sham injection of vehicle, and had no intravenous injection. However, control mice were irradiated using the same “rig” and technique as used in the MnSOD-PL injected mice. Control mice were observed for the same long-term radiation side effects as the irradiated groups. Similar statistical analysis of data was applied to all groups of control mice.

Mice were followed for their lifetime. Skin lesions were treated with Biafine cream, Ortho Neutrogena, Los Angeles, CA. No other administration of systemic antibiotics or supportive care was utilized.

Gene Therapy

MnSOD-Plasmid Liposomes were prepared according to published methods (27). The construct pNVL3-MnSOD containing the full length human MnSOD transgene was utilized (27).

In preliminary experiments, empty liposomes or the control plasmid pNVL3 had no radioprotective effect compared to irradiation alone with respect to survival at 30 days of mice after 9.5Gy total body irradiation, therefore these control groups, were eliminated from the long term study. Mice received 100 μ l of liposomes containing 100 μ g of MnSOD-PL plasmid (27). Statistical evaluation of cohorts in treatment groups to facilitate adequate power calculation for outcomes was carried out prior to the experiment. Groups of 50 mice received 9.5 Gy, 1.0 Gy, whole body irradiation, or no irradiation. Equal size groups of female and male mice in each experiment received MnSOD-PL intravenously 24 hrs prior to each irradiation dose. In previous studies we carried out experiments with intravenous (I.V.) injection of empty liposomes, scrambled sequence MnSOD inactive plasmid liposomes, and empty plasmid liposomes. These agents had no significant effect compared to irradiation alone controls with respect to the effects of 9.5 Gy at 30 days. Because of the expense of including these controls again and holding mice for the two year study, we did not include these additional control groups.

After irradiation, mice were returned to cages at 5 per cage and continued to be fed standard laboratory chow (Lab Diet 5P76 Irradiated ProLab Iso Pro RMH 3000).

Gross and Histopathologic Evaluation

Dead or dying mice were subjected to complete necropsy. The thoracic and abdominal cavities were meticulously examined. The entire small and large intestine was examined for evidence of tumors. The bone marrow, nasal cavity, upper airway, including oral cavity and oropharynx was examined. Soft tissue masses in the thorax or abdomen and normal tissues were dissected and fixed in 50% formalin/saline for normal tissue and abnormal histopathologic section evaluation. Muscles in upper and lower extremities, as well as abdomen and back were examined for evidence of muscle tumors. All mass lesions were removed and separately analyzed for histopathologic evidence of malignancy. Thymus region and thymic gland tumors were separately removed for evaluation.

Statistical Methods

Sample size of 50 animals per group was based on considerations of FDA draft guidance (29), including feasibility and cost of study implementation, and the need for a sufficient number of relatively long-term surviving animals to assess whether any increase in survival arising from treatment with MnSOD might be accompanied by a corresponding increase in late-onset cancers. Considering mice irradiated to 9.5 Gy (the estimated LD50/30), 50 animals per group provides 80% power to detect a 50% reduction in 30-day mortality (0.05 one-sided exact test) for the MnSOD-treated versus control animals of each sex. With respect to cancer developing in the animals irradiated to 9.5 Gy, we estimated that the lifetime cumulative incidence might be as high as 30% among the long-term surviving controls. For both sexes combined, we expected approximately 74 long-term survivors (≥ 31 d) among the MnSOD-treated animals as compared to 50 long-term surviving controls. Thus, we estimated our study would have 80% power (at significance level 0.10, one-sided exact test) to detect a 1.7-fold increase in overall cancer incidence among the MnSOD-treated animals. We used a reduced

standard of “significance” in this instance because our purpose is toxicity screening, i.e., deriving evidence suggestive of delayed carcinogenicity or absence thereof among the MnSOD-treated animals. With regard to our analyses of the resulting data, the estimates of overall survival are based on the Kaplan-Meier method, with differences between groups assessed by the log-rank test (30). We used the Poisson test implemented in StatXact (31) for comparing mortality- and cancer-incidence rates over specific periods of time: 0–30 d, 31d–1yr, ≥ 1 yr. For the long-term survivors, we also applied proportional hazards regression analysis for statistical modeling of overall cancer incidence in relation to three covariates: sex of the animal, dose of MnSOD, and dose level of radiation.

RESULTS

MnSOD-PL administration decreases death from the hematopoietic syndrome following total body irradiation

C57BL/6J female and male mice were divided into groups of 50 and irradiated according to the methods. Figure 1 shows survival from day 1 (start of treatment) by dose level of radiation, MnSOD-PL, and sex of animal. At 9.5 Gy irradiation, 30-day mortality was significantly greater in the female and male irradiated controls as compared to their MnSOD-PL-treated counterparts: 44% mortality in female irradiated controls vs. 10% ($p = 0.0013$) for MnSOD-PL-treated females, and 76% mortality in male irradiated controls vs. 24% ($p < 0.0001$) for MnSOD-PL-treated males (Table 1). This life-sparing effect of MnSOD-PL short term did not come at the expense of higher mortality rates later in life, as shown in Figure 2, which represents conditional survival from day 31. Among the 9.5Gy irradiated females surviving 31 days or more, the conditional median survival time was 393 days for the MnSOD-PL-treated animals as compared to 430 days for the irradiated controls ($p = 0.64$). Among the 9.5-Gy irradiated males surviving 31 days or more, the conditional median survival time was 427 days for the MnSOD-PL-treated animals as compared to 248 days for the irradiated controls ($p = 0.03$). These findings are represented graphically in Figure 2.

After 1 Gy irradiation, 30-day mortality was uniformly low in both female and male animals, and there was no significant difference in mortality rates by MnSOD-PL treatment (Table 1). The conditional median survival time for females surviving 31 days after 0 Gy irradiation, was 568 days for MnSOD-PL vs. 538 days for control irradiated ($p = 0.49$); at 1 Gy irradiation, the median survival time was 561 days for MnSOD-PL vs. 548 days for control irradiated ($p = 0.02$). In the male mice, median survival after 30 days was 229 days for 9.5 Gy irradiated mice compared to 420 days for MnSOD-PL + 9.5 Gy mice ($p = 0.0089$). Median survival was 533 or 567 days for 0 Gy and MnSOD-PL + 0 Gy, respectively, and 550 and 582 days for 1 Gy and MnSOD-PL + 1 Gy, respectively.

The increased total body radiosensitivity of male compared to female mice confirms results published previously (2). Male mice receiving MnSOD-PL intravenously prior to 9.5 Gy total body irradiation showed a survival of 74% at 30 days which was significantly increased ($p < 0.0001$). These results are consistent with the sub-lethality of the 1.0 Gy total body irradiation dose in this mouse strain, and confirm and extend results of previous publications demonstrating the increased total body radiosensitivity of male compared to female mice (18).

MnSOD-PL treatment improves long term survival from total body irradiation in mice over 350 days

All mice in both male and female experimental groups were followed for the long-term effects of ionizing irradiation. Study of the survivors of the LD 50/60 dose have been suggested as being highly relevant to determine the stability of stem cell populations in serial bone marrow

transplantation assays (32). The data in Figures 1 and 2 demonstrate a statistically significant increase in survival of 9.5 Gy total body irradiated mice receiving MnSOD-PL in both male and female groups. These results are consistent with the known radioprotective effects of MnSOD-PL on specific organs (22–28) and extend this positive effect to long-term survival after recovery from the hematopoietic syndrome.

Mice followed out past 360 days demonstrated improved survival in the MnSOD-PL treated groups in both female and male groups. Death rates from life shortening effects of total body radiation were observed in both irradiation control and MnSOD-PL treated groups in the 9.5 Gy radiation experiment.

Mice receiving 1.0 Gy total body irradiation showed no significant life shortening until 350 days. MnSOD-PL treatment showed significant improvement in survival in both female and male groups receiving this lower dose total body irradiation.

The incidence of radiation induced malignancy is not increased by MnSOD-PL radioprotective gene therapy

Table 2 provides a summary of cancer incidence (all types combined) occurring beyond 30 days in relation to radiation dose level, sex of animal, and treatment with MnSOD. (Cancers arising in animals dying within 30 days of radiation was not investigated.) Several points should be noted. First, cancer incidence in controls was relatively low and bore no relationship to radiation dose level: the incidence rate of cancer among female and male controls (combined) was 9.0 animals developing cancer per 100 animals per year at 0 Gy, 12.1 at 1 Gy, and 4.8 at 9.5 Gy. Second, the cancer incidence rate in the MnSOD-treated animals was not significantly greater than that in the controls; neither was it significantly lower. All of the differences in cancer incidence rates between the MnSOD-treated and control animals are compatible with chance: $p \geq 0.49$. Third, adjusted for radiation dose level, the estimated cancer incidence rate in the MnSOD-treated animals (males & females combined) was 1.1 times the cancer incidence rate in the controls ($p = 1.00$). Fourth, there was no trend in the relative incidence rates, i.e., the ratio of cancer incidence in MnSOD-treated vs. controls, by radiation dose level (Poisson test of trend: $p = 0.84$).

The causes of death of all the survivors of the hematopoietic syndrome in female (Table 3) and male mouse groups (Table 4) were carefully evaluated. Gross histopathologic evidence of tumors revealed an irradiation induced increased incidence of thymoma, lymphomas, soft tissue sarcoma, gastrointestinal tumors, and lung tumors that was consistent with previous published data on this inbred C57BL/6J mouse strain (18). Mice in all groups showed age related skin lesions including pruritis. These skin changes were associated with benign splenic hypertrophy also known as myeloid metaplasia (33) which has been attributed to multiple age related factors including activation of endogenous retroviruses (19) in this mouse strain.

DISCUSSION

The effects of total body or sub-total body irradiation on life shortening have been well documented in experimental model systems (18). Recent data provide evidence for common effects of radical oxygen species (ROS) in the physiology of irradiation damage; aging and carcinogenesis (12,13,16,34–37). Ionizing irradiation is known to induce rapid production of ROS, and recent data demonstrate persistent production of ROS in irradiated tissues out to six months after irradiation (32–38). Treatment with antioxidants including SOD mimetics (12) has been shown to improve a lifespan in *c-elegans*, and other animal models. Furthermore, intrinsic elevation of MnSOD in the tissues of female mice (2) is consistent with the improved total body irradiation survival of female compared to male mice. These data taken together suggest that MnSOD-PL radioprotective gene therapy might be expected to confer a short term

survival advantage. However, due to rapid clearance of the transgene, the therapy might confer no specific long term survival advantage.

Published data suggest that MnSOD-PL administration prior to irradiation, decreases long-term production of ROS, and long-term delayed production of cytokines (27). Thus, MnSOD-PL therefore, might be expected to have an effect on reducing long-term ROS production (32–38). To determine whether these possible outcomes might affect long term survival, experiments were carried out on large groups of female and male mice exposed to 9.5 Gy or 1.0 Gy total body irradiation. Some groups received MnSOD-PL administration systemically by intravenous injection 24 hrs prior to irradiation. The results indicate a significant radioprotective effect with respect to the LD 50/30, and LD 50/60 dose of total body irradiation. The improved survival in female compared to male mice receiving 9.5Gy irradiation confirm and extend previous publications on relative radioresistance of female compared to male mice (2). A statistically significant improvement in survival was detected in both female and male mice. The magnitude of protection by MnSOD-PL against 9.5Gy TBI was greater in male mice.

Preliminary experiments comparing MnSOD-PL and pNVL3 (empty plasmids)-PL showed no significant improvement in survival at 30 or 60 days with either empty liposomes or those containing control plasmid without the MnSOD transgene. These data confirm and extend previous experiment showing no radioprotection by empty liposomes or control pNVL3 plasmid liposomes as controls for lung radiation protection and esophagus radiation protection by MnSOD-PL (22–23). Because of the size required for cohort groups, the empty liposome and control pNVL3 groups were not included in the long-term studies.

MnSOD-PL has been studied for use in conferring protection against radiation induced toxicity in an organ specific fashion.(43–44) The mechanism of its protective effect as understood at the present time indicates that removal of superoxide by MnSOD-mediated dismutation to hydrogen peroxide occurs first. Decreased superoxide leads to less combination with nitric oxide and, therefore, less available product peroxynitrite.(20) Decreased peroxynitrite leads to less mitochondrial mediated apoptosis.(21) Other MnSOD-inducing radioprotective drugs including Amifostine may work by this same mechanism pathway.(42)

MnSOD levels in the intestine were not measured in the present studies. Prior data have shown prolonged MnSOD transgene product expression in vascular endothelial cells of the lung. (44) Previous publications have outlined the importance of MnSOD-PL mediated increase in protein levels prior to irradiation.(43) We did not specifically study the effects of 9.5 Gy on intestinal, induction of endogenous MnSOD. The key organ/tissue responsible for increasing survival of mice in the present studies will be approached in future experiments in which we will focus on this question.

Detection of the MnSOD transgene and its products in target organs and tissues is being carried out as part of a separate study. We are carrying out detailed studies in the next series of experiments along these lines and will measure levels of MnSOD-transgene protein in multiple organs including the intestine.

Post-irradiation treatment with MnSOD-PL protection of the lung has been studied in previous publications, and is not effective as pre-irradiation treatment.(43) Overexpression of MnSOD by MnSOD-PL does indeed decrease irradiation-induced apoptosis.(20–21) This role in anti-carcinogenesis is not known. The MnSOD-PL mediated anti-carcinogenetic effect noted by others is being studied separately.

A potential concern with respect to radioprotective gene therapy is the possible persistence of radiation damaged but surviving cells in self-renewing proliferative tissues. Such survival of damaged cells could be relatively harmful to the process of clonal succession leading to the

emergence of transformed clones that might cause leukemia/lymphoma in the hematopoietic system, or solid tumors, particularly gastrointestinal and those in other organs with a high proportion of self-renewing stem cells. A meticulous evaluation of gross and microscopic histopathology in all dead and dying mice revealed no significant increase in malignancy in those sub-groups receiving MnSOD-PL above the baseline incidence of irradiation induced tumors in C57BL/6J mice surviving total body irradiation (19). In particular, there was no increase in the incidence of known irradiation induced thymic lymphoma (19) compared to solid tissue tumors in the MnSOD-PL treated groups compared to the irradiation control groups. These data suggest that improved survival from the acute hematopoietic syndrome by antioxidant gene therapy is not associated with a detectable additional deleterious effect with respect to irradiation induced life shortening or death from malignancy (39). Concerning the MnSOD lethality-sparing effect over the long-term, there was no detectable increase in the radiation carcinogenic effect, life shortening effect, or other responses. MnSOD induction by Amifostine may have a similar effect.(42)

The present studies were carried out using a single administration of antioxidant gene therapy prior to irradiation. Other studies have suggested that repeat administration of MnSOD-PL in fractionated irradiation experiments is safe and efficacious and is associated with no further exacerbation of acute irradiation effects (33–34). However, the effects of organ specific MnSOD-PL gene therapy on long-term leukemogenesis and carcinogenesis had not been reported. We now demonstrate with systemic MnSOD-PL and TBI that there is no detectable negative effect of this gene therapy modality. We do not know the percent of marrow cells transfected by a single I.V. MnSOD-PL dose. HA-MnSOD epitope tagged plasmid-liposomes injected I.V. showed few detectable positive cells at 24 hrs in marrow, spleen and liver. However, at 24 hrs, 30 cycle-RT PCR analyses for MnSOD specific sequences showed positivity in marrow and liver but not in the intestine or muscle. The mice in the present experiments were not fed specific antioxidant diets before or after irradiation. The addition of antioxidant diets to the MnSOD-PL gene therapy protocol may further improve the long-term outcome after irradiation and may provide further decrease in the observed irradiation induced life shortening (39).

Using MnSOD-PL as a counter measure for first responders in a radiation counterterrorism effort would be limited. There were marginal decreases in radioprotection, and there was a necessity of using I.V. administration at this stage, both of which are problematic issues.(40–41)

The present results provide support for use of MnSOD-PL administration as one of many possible radiation counter measures for the treatment of first responders in a radiation accident or terrorism event. The data suggest that administration of MnSOD-PL and possibly other radioprotective antioxidant agents prior to irradiation will not have a deleterious late effect on the expected enhanced number of survivors from the hematopoietic syndrome. The addition to MnSOD-PL treatment of other radiation mitigating agents immediately or continually after irradiation may further improve survival.

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References

1. Naugler W, Willscott E, Sakurai T, Kim S, Maeda S, Kim K, Eisharkawy A, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-Dependent IL-6 production. *Science* 2007;317:121–124. [PubMed: 17615358]

2. Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo F, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radical Biology & Medicine* 2003;34(5):546–552. [PubMed: 12614843]
3. Matheu A, Maraver A, Klatt P, Flores I, Garcia-Cao I, Borras C, Flores J, Vina J, Blasco M, Serrano M. Delayed aging through damage protected by the Arf/p53 pathway. *Nature* 2007;448(19):375–380. [PubMed: 17637672]
4. Toren F, Manuel S, Maria B. The common biology of cancer and ageing. *Nature* 2007;448:767–771. [PubMed: 17700693]
5. Pinton P, Rimessi A, Marchi S, Orsini F, Migliaccio E, Giorgio M, Contursi C, Minucci S, Mantovani F, Wieckowski M, Del Sal G, Pelicci Pier G, Rizzuto R. Protein kinase C β and Prolyl Isomerase 1 regulate mitochondrial effects of the life-span determinant p66. *Science* 2007;315:659–662. [PubMed: 17272725]
6. Dasari A, Bartholomew J, Volonte D, Galbiati F. Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements. *Cancer Res* 2006;66(22):10805–10814. [PubMed: 17108117]
7. Rottiers V, Motola D, Daniel L, Gerisch B, Cummins C, Nishiwaki K, Mangelsdorf D, Antebi A. Hormonal control of *C. elegans* dauer formation and life span by a rieske-like oxygenase. *Developmental Cell* 2006;10:473–482. [PubMed: 16563875]
8. An Jae H, Vranas K, Lucke M, Inoue H, Hisamoto N, Matsumoto K, Blackwell K. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *PNAS* 2005;102(45):16275–16280. [PubMed: 16251270]
9. Sanchez D, Lopez-Arlas B, Torroja L, Canal I, Wang X, Bastiani M, Ganfornina M. Loss of glial lazaro, a homolog of apolipoprotein D, reduces lifespan and stress resistance in *Drosophila*. *Current Biology* 2006;16:680–686. [PubMed: 16581513]
10. Rea Shane L, Wu D, Cypser J, Vaupel J, Johnson T. A stress-sensitive reporter predicts longevity in isogenic populations of *Caenorhabditis elegans*. *Nature Genetics* 2005;17(8):894–898. [PubMed: 16041374]
11. Lehtinen M, Yuan Z, Boag P, Yang Y, Villen J, Becker E, DiBacco S, de la Iglesia N, Gygi S, Blackwell T, Bonni A. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* 2006;125:987–1001. [PubMed: 16751106]
12. Simon S, Ravenscroft J, Malik S, Gill M, Walker D, Clayton P, Wallace D, Malfroy B, Doctrow S, Lithgow G. Extension of life-span with superoxide dismutase/catalase mimetics. *Science* 2000;289:1567–1571. [PubMed: 10968795]
13. Serrano M, Blasco M. Cancer and ageing: convergent and divergent mechanisms. *Nature Reviews Molecular Cell Biology* 2007;8:715–721.
14. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nature Reviews Molecular Cell Biology* 2007;8:729–734.
15. Sharpless N, DePinho R. How stem cells age and why this makes us grow old. *Nature Reviews Molecular Cell Biology* 2007;8:703–713.
16. Finkel T, Serrano M, Blasco M. The common biology of cancer and ageing. *Nature* 2007;448:767–774. [PubMed: 17700693]
17. Ball C, Pilz I, Schmidt M, Fessler S, Williams D, von Kalle C, Glimm H. Stable differentiation and clonality of murine long-term hematopoiesis after extended reduced-intensity selection for MGMT P140K transgene expression. *Blood* 2007;110:1779–1784. [PubMed: 17496202]
18. Klein, J. *Principles of Immunogenetics Applied to a Single System*. Springer-Verlag; New York, NY: 1975. *Biology of the mouse histocompatibility-2 complex*.
19. Gross, L. *Oncogenic Viruses*. 2. Pergamon Press; New York, NY: 1970.
20. Epperly MW, Sikora C, Defilippi S, Gretton J, Zhan Q, Kufe DW, Greenberger JS. MnSOD inhibits irradiation-induced apoptosis by stabilization of the mitochondrial membrane against the effects of SAP kinases p38 and Jnk1 translocation. *Radiat Res* 2002;157:568–577. [PubMed: 11966323]
21. Epperly MW, Gretton JE, Bernarding M, Nie S, Rasul B, Greenberger JS. Mitochondrial localization of copper/zinc superoxide dismutase (Cu/ZnSOD) confers radioprotective functions in vitro and in vivo. *Radiat Res* 2003;160:568–578. [PubMed: 14565825]

22. Carpenter M, Epperly MW, Agarwal A, Nie S, Hricisak L, Niu Y, Greenberger JS. Inhalation delivery of manganese superoxide dismutase-plasmid/liposomes (MnSOD-PL) protects the murine lung from irradiation damage. *Gene Therapy* 2005;12:685–690. [PubMed: 15750616]
23. Stickle RL, Epperly MW, Klein E, Bray JA, Greenberger JS. Prevention of irradiation-induced esophagitis by plasmid/liposome delivery of the human manganese superoxide dismutase (MnSOD) transgene. *Radiat Oncol Invest Clinical & Basic Res* 1999;7(6):204–217.
24. Guo HL, Seixas-Silva JA, Epperly MW, Gretton JE, Shin DM, Greenberger JS. Prevention of irradiation-induced oral cavity mucositis by plasmid/liposome delivery of the human manganese superoxide dismutase (MnSOD) transgene. *Radiat Res* 2003;159:361–370. [PubMed: 12600239]
25. Kanai AJ, Zeidel ML, Lavelle JP, Greenberger JS, Birdler LA, deGroat SC, Apodaca GL, Meyers SA, Ramage R, Epperly MW. Manganese superoxide dismutase gene therapy protects against irradiation-induced cystitis. *Am J of Physiology (Renal Physiology)* 2002;44:1152–1160.
26. Guo HL, Wolfe D, Epperly MW, Huang S, Liu K, Glorioso JC, Greenberger JS, Blumberg D. Gene transfer of human manganese superoxide dismutase protects small intestinal villi from radiation injury. *J Gastrointest Surg* 2003;7:229–236. [PubMed: 12600447]
27. Epperly MW, Gretton JA, Defilippi SJ, Sikora CA, Liggitt D, Koe G, 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* 2001;155:2–14. [PubMed: 11121210]
28. Epperly MW, Guo HL, Jefferson M, Wong S, Gretton J, Bernarding M, Bar-Sagi D, Greenberger JS. Cell phenotype specific duration of expression of epitope-tagged HA-MnSOD in cells of the murine lung following intratracheal plasmid liposome gene therapy. *Gene Therapy* 2003;10:163–171. [PubMed: 12571645]
29. Center for Drug Evaluation and Research. Statistical Aspects of the Design, Analysis, and Interpretation of Chronic Rodent Carcinogenicity. <http://www.fda.gov/cder/guidance/815dft.htm>
30. Klein, JP.; Moeschberger, ML. *Survival Analysis*. 2. Springer; New York: 2003.
31. StatXact 8 Overview, Cytel Statistical Software and Services, <http://www.cytel.com/Products/StatXact/Default.asp>.
32. Greenberger JS, Epperly MW. Radioprotective antioxidant gene therapy: Potential mechanisms of action. *Gene Ther Mol Biol* 2004;8:31–44.
33. Greenberger JS, Stephenson JR, Moloney WC, Aaronson SA. Different hematologic diseases induced by Type-C viruses chemically activated from embryo cells of different mouse strains. *Cancer Res* 1975;35:245–252. [PubMed: 45891]
34. Spitz DR, Azzam EL, Li JJ. Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: A unifying concept in stress response biology. *Cancer Metastasis Rev* 2004;23:311–322. [PubMed: 15197331]
35. Mitchell JB, Krishna MC. Nitroxides as radiation protectors. *Mil Med* 2002;167:49–50. [PubMed: 11873514]
36. Hahn SM, Krishna MC, DeLuca AM, Coffin D, Mitchell JB. Evaluation of the hydroxylamine tempol-H as an in vivo radioprotector. *Free Radic Biol Med* 2000;28:953–958. [PubMed: 10802227]
37. Weiss JF, Landauer MR. Radioprotection by antioxidants. *Ann N Y Acad Sci* 2000;899:44–60. [PubMed: 10863528]
38. Greenberger JS, Epperly MW, Gretton J, Jefferson M, Nie S, Bernarding M, Kagan V, Guo HL. Radioprotective gene therapy. *Current Gene Therapy* 2003;3:183–195. [PubMed: 12762478]
39. Rotblat J, Lindop P. Long-term effects of a single whole-body exposure of mice to ionizing radiations. II Causes of death. *Proc R Soc Lond [Biol]* 1961;154:350–368.
40. Stone HB, Moulder JE, Coleman CN, Ang KK, Anscher MS, Barcellos-Hoff MH, Dynan WS, Fike JR, Grdina DJ, Zaharevitz D. Models for evaluating agents intended for the prophylaxis, mitigation, and treatment of radiation injuries. Report of an NCI workshop, December 3–4, 2003. *Rad Res* 2004;162:711–728.
41. J. W. Poston. Commentary No. 19 – Key elements of preparing emergency responders for nuclear and radiological terrorism. *N.C.R.P.* pg. 1–67 (2005).
42. Carnes BA, Grdina DJ. In vivo protection by the aminothiols WR-2721 against neutron-induced carcinogenesis. *Int J of Radiation Biology* 1992;61:567–576.

43. Epperly MW, Guo HL, Bernarding M, Gretton J, Jefferson M, Greenberger JS. Delayed intratracheal injection of manganese superoxide dismutase (MnSOD)-plasmid/liposomes provides suboptimal protection against irradiation-induced pulmonary injury compared to treatment before irradiation. *Gene Ther Mol Biol* 2003;7:61–68.
44. Epperly MW, Guo HL, Jefferson M, Wong S, Gretton J, Bernarding M, Bar-Sagi D, Greenberger JS. Cell phenotype specific duration of expression of epitope-tagged HA-MnSOD in cells of the murine lung following intratracheal plasmid liposome gene therapy. *Gen Therapy* 2003;10:163–171.

Figure 1 A

Figure 1 B

Figure 1. Survival from Day 1, by MnSOD-PL Treatment and Radiation Dose Level

C57BL/6J female or male mice were divided into two groups of 150 mice each. One group was injected with MnSOD-PL intravenously. 50 mice from each group were not irradiated. Another 50 mice from each group were administered 1 Gy whole body irradiation with the remaining 50 mice in each group administered 9.5 Gy whole body irradiation 24 hr after injection of MnSOD-PL. The mice were then followed for survival and sacrificed when appearing moribund. The control group was irradiated with no sham injection. Controls were handled exactly as were the injected mice.

Figure 2A

Figure 2B

Figure 2. Conditional Survival from Day 31, by MnSOD-PL Treatment and Radiation Dose Level
 Conditional survival of all mice surviving the first 30 days which was the time of death due to hematopoietic failure was determined on the surviving mice from the six groups from Figure 1. The mice were sacrificed when appearing moribund. The control group was irradiated with no sham injection. Controls were handled exactly as were the injected mice.

Table 1
Mortality in Relation to Radiation Dose, Sex of Animal, and MnSOD Treatment

Irradiation Dose	Sex	MnSOD (µg)	30 Day Mortality		Survival Past 30 Days*	
			Percent	P**	Median	P***
0 Gy	Females	0	0	1.00	538	0.49
	Males	100	0	0.49	568	0.75
1.0 Gy	Females	0	4	1.00	553	0.02
	Males	100	0	0.24	567	0.52
9.5 Gy	Females	0	6	0.0013	550	0.64
	Males	100	44	<0.0001	430	0.03
			10		393	
			76		229	
		100	24		420	

12.□ Analysis for animals surviving more than 30 Days

** Fisher's Exact Test
*** Log Rank Test

Table 2 Cancer Incidence in Relation to Radiation Dose, Sex of Animal and MnSOD Treatment

Irradiation Dose	Sex	MnSOD (µg)	All Cancers			
			N [*]	TDAR ^{**}	Rate ^{***}	P ^{****}
0 Gy	Females	0	5	18294	10	1.00
		100	4	14516	10.1	
	Males	0	0	2082	0	1.00
1 Gy		100	0	1969	0	
	Combined	0	5	20376	9.0	1.00
		100	4	16485	8.9	
1 Gy	Females	0	6	18322	12.0	0.49
		100	2	11891	6.1	
	Males	0	1	2853	12.8	1.00
9.5 Gy		100	0	1328	0	
	Combined	0	7	21175	12.1	0.50
		100	2	13219	5.5	
9.5 Gy	Females	0	1	6568	5.6	1.00
		100	2	7206	10.1	
	Males	0	0	696	0	1.00
9.5 Gy		100	2	4621	15.8	
	Combined	0	1	7537	4.8	0.64
		100	4	11827	12.3	

* N = Number of Tumors
** TDAR = Total Days at Risk for Cancer
*** Rate = Cancer Incidence Rate
**** P = Poisson Test

Table 3

Gross and Microscopic Histopathologic Findings in Irradiated Dying C57BL/6J Female Mice

Group	Day after Irradiation	Histopathologic Findings
0 Gy	357	Lymphoma
	385	Lung fibrosis
	385	Lymphoma
	538	Salivary Gland Hyperplasia
	546	Lymphoma
	546	Myeloid Metaplasia
	554	Lymphoma
	560	Ovarian Cyst
	566	Nasal Abscess
	571	Ovarian Adenocarcinoma
	599	Lymphoma
	607	Ovarian Cyst + Myeloid Metaplasia
	621	Lymphoma
	642	Uterine Sarcoma
0 Gy + MnSOD-PL	357	Extra Medullary Hematopoiesis
	357	Myeloid Metaplasia
	417	Myeloid Metaplasia
	417	Nasal Abscess
	434	Myeloid Metaplasia
	434	Lymphoma
	438	Fibrosis of tail
	508	Lymphoid Hyperplasia
	571	Lymphoma + Extra Medullary Hematopoiesis
	573	Lymphoid Hyperplasia
	574	Renal Cyst with Lymphoid Infiltrate
	574	Intestinal Abscess
	574	Salivary Gland Hypertrophy
	576	Lymphoid Hyperplasia
	593	Ovarian Cyst
	607	Fecal Impaction
	614	Lymphoma
	614	Lymphoma
	690	Lymphoma
	713	Lymphoma
1 Gy	275	Lymphoma
	387	Ovarian Cyst
	486	Myeloid Metaplasia
	486	Ovarian Cyst
	510	Myeloid Metaplasia
	548	Lymphoma
	548	Lymphoma
	556	Myeloid Metaplasia
	562	Lymphoma
	562	Myeloid Metaplasia
	568	Lymphoma
	568	Lymphoid Hyperplasia
	573	Lymphoma
	588	Lymphoid Hyperplasia
	595	Lymphoma
	601	Hematoma
	653	Lymphoma
	715	Lymphoma
1 Gy + MnSOD-PL	359	Nasal Abscess
	461	Myeloid Metaplasia
	461	Multifocal Hepatic Neurosis
	471	Myeloid Metaplasia
	471	Intestinal Abscess
	513	Salivary Gland Hyperplasia
	523	Adenocarcinoma of Stomach
	573	Lymphoid Hyperplasia
	573	Cervical Abscess
	601	Lymphoma
	601	Pneumonia
	630	Ovarian Cyst
	644	Lymphoma
	692	Ovarian Adenocarcinoma
9.5 Gy	243	Myeloid Metaplasia
	275	Extramedullary Hematopoiesis
	325	Thymoma
	348	Focal Hepatic Necrosis

Group	Day after Irradiation	Histopathologic Findings
	550	Cervical Abscess
9.5 Gy + MnSOD-PL	233	Cervical Sarcoma
	358	Thymoma
	358	Intestinal Fibrosis
	407	Lung Fibrosis
	407	Myeloid Metaplasia

Extra Medullary Hematopoiesis = liver hematopoiesis and increased spleen size with hematopoiesis

Myeloid Metaplasia = spleen only enlargement

Table 4**Gross and Microscopic Histopathologic Findings in Irradiated Dying C57BL/6J Male Mice**

Group	Day After Irradiation	Histopathologic Findings
0 Gy	440	Myeloid Metaplasia
	448	Hematoma
	469	Pneumonia
	547	Lymphoma
0 Gy + MnSOD-PL	434	Myeloid Metaplasia
	469	Lymphoma
	531	Lymphoma
1 Gy	393	Lymphoma
	393	Hematoma (abdomen)
	399	Oral Cavity Abscess
	446	Myeloid Metaplasia
	481	Lymphoma
	505	Myeloid Metaplasia
	529	Hematoma
	540	Myeloid Metaplasia
	543	Infection
	545	Infection
1 Gy + MnSOD-PL	505	Infection
	533	Hematoma
	540	Lymphoma
9.5 Gy		
9.5 Gy + MnSOD-PL	142	Adenocarcinoma of Pancreas
	142	Thymoma
	279	Nasal Abscess
	378	Lymphoma
	455	Lymphoid Infiltrate
	462	Inflammation
	486	Inflammation
	531	Myeloid Metaplasia
	563	Lymphoma

Extra medullary hematopoiesis = liver hematopoiesis and spleen increased size with hematopoiesis

Myeloid Metaplasia = spleen only enlargement