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*Radiat Res*. Author manuscript; available in PMC 2011 April 1.

Published in final edited form as:

*Radiat Res*. 2010 April ; 173(4): 453–461. doi:10.1667/RR1763.1.

# Irradiated Esophageal Cells are Protected from Radiation-Induced Recombination by MnSOD Gene Therapy

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## Abstract

Radiation-induced DNA damage is a precursor to mutagenesis and cytotoxicity. During radiotherapy, exposure of healthy tissues can lead to severe side effects. We explored the potential of mitochondrial SOD (MnSOD) gene therapy to protect esophageal, pancreatic and bone marrow cells from radiation-induced genomic instability. Specifically, we measured the frequency of homologous recombination (HR) at an integrated transgene in the Fluorescent Yellow Direct Repeat (FYDR) mice, in which an HR event can give rise to a fluorescent signal. Mitochondrial SOD plasmid/liposome complex (MnSOD-PL) was administered to esophageal cells 24 h prior to 29 Gy upper-body irradiation. Single cell suspensions from FYDR, positive control FYDR-REC, and negative control C57BL/6NHsd (wild-type) mouse esophagus, pancreas and bone marrow were evaluated by flow cytometry. Radiation induced a statistically significant increase in HR 7 days after irradiation compared to unirradiated FYDR mice. MnSOD-PL significantly reduced the induction of HR by radiation at day 7 and also reduced the level of HR in the pancreas. Irradiation of the femur and tibial marrow with 8 Gy also induced a significant increase in HR at 7 days. Radioprotection by intraesophageal administration of MnSOD-PL was correlated with a reduced level of radiation-induced HR in esophageal cells. These results demonstrate the efficacy of MnSOD-PL for suppressing radiation-induced HR *in vivo*.

## Introduction

Therapeutic doses of ionizing radiation are often associated with excessive toxicity in the esophagus, thus limiting the dose that can be delivered to the tumor as well as creating potentially lethal side effects for the patient. Extensive studies of radiation-induced tissue damage in mice have yielded new avenues for therapeutic advances. In the mouse, ionizing radiation delivered to the esophagus is associated with epithelial cell apoptosis, microulceration, and the clinical syndrome of dysphagia, dehydration and weight loss (1–4). These toxic effects are thought to be mediated not only by the direct effects of radiation-induced ionizations but also by ROS generated by ionization of water as well as ROS that are created by immune cells during the resulting inflammatory response. To suppress the toxic effects of radiation, gene therapy approaches are being developed to deliver proteins that suppress ROS toxicity.

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One strategy for reducing the levels of potentially toxic ROS is to supplement tissues with proteins that potentiate clearance. In the case of superoxide, clearance can be accelerated by increasing the levels of superoxide dismutases. Numerous studies have demonstrated the efficacy of such an approach using MnSOD plasmid liposomes (MnSOD-PL). For example, intraesophageal administration of MnSOD-PL reduces esophageal epithelial damage and improves migration of restorative bone marrow progenitors to the esophageal squamous epithelium (1,4–6). MnSOD-PL administration also reduces esophageal cell lipid peroxidation (7–9) and improves the survival of esophageal somatic stem cells (8,10,11). Furthermore, MnSOD-PL prevents longer-term tissue damage, as seen by the observation that MnSOD suppresses late esophageal stricture after irradiation (3,11). Finally, MnSOD-PL treatment reduces pro-inflammatory cytokines in the esophagus (3). Taken together, these results indicate that gene therapy using MnSOD-PL protects against radiation-induced tissue damage and suppresses radiation-induced inflammation, suggesting that expression of the MnSOD transgene reduces the levels of ROS.

In addition to immediate cell and tissue damage, the risk of secondary cancers is a general concern with therapeutic radiation. DNA is the key molecular target for such cancers, and radiation-induced DNA damage can lead to mutations that promote cancer. Most of the effects of radiation on DNA can be traced back to ROS that create a myriad of mutagenic base lesions as well as single-and double-strand breaks that promote large-scale DNA sequence rearrangements (12,13). ROS generated during inflammation are similarly genotoxic. Activated immune cells have been shown to induce mutations in human cells (14). Given that therapeutic radiation delivered to the esophagus induces high levels of ROS, one potential benefit of MnSOD-PL might be to suppress radiation-induced changes in DNA sequences in the esophagus.

To explore the possibility that MnSOD-PL might protect against radiation-induced changes in DNA sequences, we exploited a transgenic mouse model in which large-scale DNA sequence rearrangements in an integrated transgene can be detected. Fluorescent Yellow Direct Repeat (FYDR) mice harbor two nonfunctional copies of expression cassettes for enhanced yellow fluorescent protein (EYFP). Sequence rearrangements promoted by homologous recombination (HR) can restore full-length coding sequence and thus give rise to a fluorescent signal (15). FYDR mice demonstrate an age-related increase in HR in the pancreas, and embryonic stem cells harboring analogous transgenes show HR in response to chemotherapeutic alkalating agents (16,17). In the present studies, we measured the effect of thoracic ionizing radiation on HR in the FYDR mouse esophagus compared to the pancreas and femoral bone marrow. The data provide evidence that ionizing radiation induces HR in the irradiated esophagus *in vivo* and that recombination events are suppressed by MnSOD-PL antioxidant gene therapy.

## Materials and Methods

### Mice and Animal Care

Male and female FYDR mice, positive control FYDR-REC mice (15,16), and wild-type C57BL/6NHsd mice were housed five per cage and fed standard laboratory chow. The mice weighed 20–23 g and were 6–8 weeks of age. FYDR-rec mice demonstrate an embryonic recombination at the FYDR locus such that tissues of adult mice have a high intrinsic level of expression of the EYFP protein and display a relatively high percentage of yellow cells (18). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh.

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### Irradiation

Unanesthetized FYDR and C57BL/6NHsd mice received intraesophageally 100 μl of water followed by 100 μg of MnSOD plasmid-liposome complexes (MnSOD-PL) in a volume of 100 μl as described previously (1). Twenty-four hours later, the MnSOD-PL-treated and control untreated mice were irradiated to the thorax with 29 Gy using a 6 MV photon beam from a Varian Clinac 23EX Linear Accelerator as described previously (1). The lower body and head were shielded such that only the thorax was irradiated. Thermoluminescence dosimeters were placed surgically in groups of five anesthetized mice on the surface of the pancreas and on the anterior surface of the femur to calculate the doses to these organs. The pancreas received *6* Gy and the femoral bone marrow 4 Gy. Previous studies documented the radioprotective effect of MnSOD-PL and the lack of detectable protection in mice receiving empty liposomes or liposomes containing either LAC-Z or CU/ZnSOD-plasmid liposomes (1,3,4). In those studies, the control groups showed no difference in radiation-induced histopathological or inflammatory change*s* (1–4). In preliminary experiments, empty liposome-treated FYDR mice, like C57BL/6 mice, showed no protection from 29 Gy upper-body irradiation; therefore, an empty liposome control group was not included for the cell sorting studies.

In a separate experiment, FYDR, FYDR-rec and C57BL/6NHsd mice were injected intravenously with 100 μl of MnSOD-PL containing 100 μl of plasmid DNA. Twenty-four hours later, the MnSOD-PL-injected and control mice were anesthetized using Nembutal and the right rear leg was irradiated with either 8 or 29 Gy. Mice irradiated with 8 Gy were killed humanely on day 0, 7 or 14 and the mice irradiated with 29 Gy were killed on day 1, 2, 3 or 7, and the bone marrow was removed. The percentages of $YFP^+$ cells were determined by flow cytometry.

### Analysis of Single Cell Preparations for Homologous Recombination

Groups of mice were killed on day 0, 7 and 14 after 29 Gy irradiation of the upper body, including the entire esophagus. The entire esophagus was dissected from the cervical esophageal/oral cavity junction down to the gastroesophageal junction. In addition, the pancreas and the bone marrow from the rear legs were harvested. Esophageal and pancreatic tissue from individual mice was minced using forceps and scissors in individual petri dishes containing 0.2% collagenase, 240 U dispase, and 0.1% trypsin followed by incubation at 37° C for 60 min for the esophagus (11) and 20 min for the pancreas (16). The entire organ containing all cell phenotypes including acinar, ductal, islet and endothelial cells was used. Single cell suspensions from bone marrow were prepared by drawing cells through successively smaller gauge needles and filtered through 100-μm filters and then 40-μm filters. The cells were washed and resuspended in 100 μl of cold HBSS containing 2% FCS and 10 m*M* Hepes buffer. The esophagus and pancreas cells were stained with anti-CD45-PE and anti-Ter119-PE-PC7 to remove contaminating hematopoietic cells. The bone marrow cells were stained with anti-CD45-PE. Propidium iodide (2 μg/ml) was added to identify dead cells. Negative control cells were isolated from the same tissues of irradiated or unirradiated C57BL/6NHsd mice. Positive control cells from each tissue were obtained from FYDR-rec mice. The cells were examined for expression of YFP using a BD FACSAria high-speed sorter (BD Biosciences, San Jose, CA). The gate for YFP-positive cells was adjusted to detect less than 1 per $4 \times 10^6$ false-positive autofluorescent cells (16). Measurements were made on individual mice from groups of male and female mice irradiated on the same day and were age-matched with irradiated FYDR-REC and negative control C57BL/HNsd (wild type) mice. Side scatter granularity (SSC-A) was plotted as a function of expression of yellow fluorescent protein (YFP-A).

The numbers of positive cells per sample were adjusted for the number of yellow-positive cells per $10^6$ cells sorted.

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In separate experiments, the bone marrow was removed from the irradiated or control unirradiated legs at 0, 1, 2, 3 and 7 days after 29 Gy and 0, 7 or 14 days after 8 Gy. Single cell suspensions and cell analysis were performed as described above. To determine whether systemic delivery of MnSOD-PL reduced HR in bone marrow, C57BL/6NHsd and FYDR mice were injected intravenously with MnSOD-PL and irradiated 24 h later with 8 Gy to the right rear leg. Seven days later, the marrow from the irradiated and unirradiated leg was analyzed for HR as described above.

### Statistics

Significant differences ($P < 0.05$) between the different treatment groups were determined using a Kruskal-Wallis test followed by a Mann-Whitney *U* test; *P* values were Bonferroni test adjusted.

## Results

### Ionizing Radiation-Induced Increase in HR in Mouse Esophageal Epithelium

FYDR mice carry a direct repeat recombination substrate in which an HR event between two mutant EYFP expression cassettes can restore full-length sequence and thus give rise to a fluorescent cell. Spontaneous HR at the FYDR transgene is a rare event [1 to 10 per million (16)]. Flow cytometry is an effective approach for detecting and quantifying these rare fluorescent cells (15). Although FYDR mice have been used for studies of cutaneous tissue and pancreatic tissue (15,17,18), HR had not been studied in the esophagus of these mice. To establish flow cytometry parameters that are effective for quantifying rare fluorescent recombinant cells, single cell suspensions of esophageal cells from negative control (C57BL/6NHsd) and positive control (FYDR-Rec) mice were analyzed. A gate was designated to include most positive yellow fluorescent cells while stringently excluding all negative control cells. Consistent with this parameter, less than one cell from the wild-type mice fell within the P5 region among the 1 million cells that were analyzed (Fig. 1A), while a significant number of cells from the positive control FYDR-Rec sample fell within the P5 gate (Fig. 1B). When single cell suspensions of esophageal tissues from an FYDR mouse were analyzed, rare fluorescent recombinant cells were captured within the P5 gate (Fig. 2C).

To measure the spontaneous frequency of recombinant cells in the esophagus, we analyzed tissue from 30 untreated FYDR mice. As expected based upon previous studies (15), the frequency of recombinant cells per sample varied from mouse to mouse (see Fig. 2, 0 Gy), with a few samples showing very high frequencies. To avoid emphasis on outliers, we tabulated the range between the first and the third quartile (the interquartile range, IQR). For untreated FYDR mice, the IQR for the esophagus ranged from 0 to 1.6 yellow cells per million cells analyzed (Table 1). This frequency is somewhat lower than what has been observed for pancreas (18), but it is similar to what has been observed for cutaneous tissue (15,18). We also analyzed pancreatic tissue and bone marrow. For both of these tissues, the median frequency was close to 0 recombinant cells per million. This was somewhat unexpected for the pancreas, because previous studies shown that the median frequency was close to 10 per million (16, 17). It is possible that variations in the sensitivity of flow cytometry between laboratories could account for this difference. For bone marrow, the observation that there were no detectable fluorescent cells in ~90% of the samples is consistent with previous studies showing that the expression of EYFP from the FYDR transgene is low in bone marrow (approximately 3% of the bone marrow cells from positive control FYDR-Rec mice are fluorescent yellow; Table 2).

Pancreas tissue from nonirradiated FYDR mice demonstrated a baseline with a median of 0.0 with an interquartile range of 0 to 4.1 positive cells per $10^6$ cells compared to esophagus, which had a median of 0.0 and an interquartile range of 0 to 1.6 (see Table 1). Bone marrow cells

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from the same FYDR mice showed low levels of HR with less than 1 per $10^6$ cells positive. These results confirm previous studies showing the background level of HR in the pancreas from these mice (18). The relatively high level of EYFP expression in the pancreas compared to other organs of FYDR-REC mice is consistent with data published previously (18). Cells from wild-type mouse esophagus, pancreas and bone marrow had almost no detectable cells that fell in the P5 region designated as $YFP^+$.

## Thoracic Irradiation Induces an Increase in HR in the FYDR Mouse Esophagus

At day 7 after 29 Gy irradiation, there was a significant increase in the frequency of recombinant cells in the FYDR mouse esophagus (compare 29 Gy and 0 Gy in Fig. 2A). Recombination was also assessed in the pancreas; while there appeared to be an increase in the frequency of recombinant cells, the increase was not statistically significant. Based on TLD measurements, the scatter dose to the pancreas from the 29-Gy exposure to the esophagus was approximately 6 Gy. We also assessed the femoral bone marrow, which received approximately 4 Gy from scatter; there was not a significant increase in the number of recombinant cells (Fig. 1C). Taken together, these data showed that 29 Gy to the esophagus induced HR, whereas the associated scatter radiation did not induce detectable HR in either the pancreas or the bone marrow.

## Intraesophageal MnSOD-PL Administration Protects the Esophagus from Ionizing Radiation-Induced HR

Previous studies showed that administration of MnSOD-PL suppresses the levels of radiation-induced apoptosis in the esophagus 7 days after irradiation (10). To test the possibility that MnSOD-PL could provide protection against radiation-induced HR, nonanesthetized FYDR mice were given intraesophageal MnSOD-PL 24 h prior to irradiation. After 7 days, single cell suspensions from individual specimens of esophagus, pancreas and bone marrow were analyzed for yellow cells. MnSOD-PL significantly reduced the level of HR in the irradiated FYDR mouse esophagus (see Fig. 2A; Table 1). MnSOD-PL treatment also significantly suppressed HR in the pancreas (Fig. 2B and Table 1). These results establish that MnSOD-PL suppresses ionizing radiation-induced sequence rearrangements in the esophagus in vivo. Although there is a significant suppression of HR in the pancreas when comparing exposed animals to exposed animals given MnSOD, we did not observe a statistically significant difference in the frequency of HR in irradiated animals compared to unirradiated control animals, leading to some ambiguity in these results.

## Increased HR in FYDR Mouse Bone Marrow Irradiated with 8 Gy

As described above, there was no detectable increase in HR in the bone marrow of FYDR mice that received 29 Gy to the esophagus and 4 Gy of scattered radiation to the bone marrow. Previous studies showed radiation-induced HR in FYDR mice exposed to 7.5 Gy (17). We therefore explored the possibility that HR might be induced in bone marrow exposed to higher doses of radiation (8 and 29 Gy). At day 7 there was a significant increase in the number of $YFP^+$ cells in the marrow of FYDR mice irradiated with 8 Gy (Table 2 and Fig. 3A).

We next explored the possibility that MnSOD might protect bone marrow from radiation-induced HR. Systemic delivery by intravenous injection of MnSOD-PL 24 h prior to irradiation did not significantly reduce the level of increase in homologous recombination in the marrow irradiated with 8 Gy (Fig. 3C, Table 3).

The 8-Gy dose to marrow was chosen to produce a level of radiation-induced apoptosis in hematopoietic cells comparable to that seen in irradiated esophagus epithelial cells after 29 Gy. We also evaluated marrow irradiated with 29 Gy, a dose that was expected to destroy all *in situ* hematopoietic repopulating cells. Mice irradiated with 29 Gy to the right rear leg did not show significant increases in the numbers of $YFP^+$ recombinant cells (Table 2 and Fig.

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3B). As expected, there were no changes in the number of $YFP^+$ cells in the marrow of irradiated positive control FYDR-rec mice at any time (Table 2 and Fig. 3), which is consistent with a lack of radiation-induced alterations in the expression of EYFP protein.

Taken together, these results show that administration of MnSOD-PL 24 h prior to irradiation significantly suppresses radiation-induced HR in the esophagus and the pancreas but not in the bone marrow. Double-strand breaks not only promote sequence rearrangements such as those promoted by HR but also act as a signal to trigger apoptosis. Therefore, the results presented here support a model in which MnSOD suppresses genotoxicity that might otherwise result in cell death or mutations.

## Discussion

Ionizing radiation induces rapid production of ROS, including superoxide. Reactive nitrogen species (RNS) are also present at sites of irradiation as a result of activated immune cells (19–21). The capacity of cells to neutralize ROS and RNS through maintenance of an antioxidant pool has been shown to be critical for cell-, tissue- and organ-specific radiation tolerance (20,21). The two major defenses against ROS and RNS stress are scavengers, such as glutathione, and antioxidant enzymes, such as superoxide dismutase (SOD). Three forms of superoxide dismutase facilitate reduction of the levels of radiation-induced superoxide. One of these, SOD2, or mitochondrial SOD (MnSOD), has been shown to ameliorate radiation-induced injury through targeted localization to the mitochondrial membrane (22,23).

Organ-specific up-regulation of MnSOD through targeted MnSOD-PL gene therapy has been shown to confer tissue- and organ-specific radioprotection in the lung, oral cavity, bladder and esophagus (23–25). The MnSOD gene therapy-mediated reduction in histopathology and tissue injury (as seen by reduced functionality) has been shown to correlate with increased levels of antioxidant capacity in individual cells (21) and decreased lipid peroxidation in irradiated tissues (9,20). Whether MnSOD-PL organ-specific gene therapy affects radiation-induced DNA sequence rearrangements *in vivo* had not been evaluated previously.

In the present studies, we delivered 29 Gy radiation to the upper body of FYDR mice and measured the levels of HR, which reflects the repair of DNA double-strand breaks, in explanted esophageal epithelial cells. In our previous studies with the same background mouse strain (C57BL/6HNsd), MnSOD-PL protection was observed at 29 Gy (1,3). In the present studies, 29 Gy induced a significant increase in HR in esophageal epithelial cells 7 days after irradiation, the time when significant apoptosis and lipid peroxidation are detected (1,9,24,25). These results confirm and extend previous studies documenting the value of the FYDR mouse strain for quantifying HR *in vivo* (16,18,26–28).

Radiation both directly ionizes the DNA and reacts with water to create ROS. Many different types of DNA lesions are created by direct ionization, ROS or RNS associated with inflammation, including base damage and single- and double-strand breaks (12,27). Among these, double-strand breaks are thought to be the most deleterious lesions, because they can be highly toxic and can lead to large-scale sequence rearrangements through HR events (28–31). HR can lead to insertions, deletions, translocations and LOH, all of which have been shown to promote cancer (29–31). Therefore, the levels of HR reflect the extent to which cells have increased levels of cancer-promoting sequence rearrangements. Since radiation-induced sequence changes are one of the most serious long-term consequences of radiation exposure, strategies to suppress such rearrangements would be of great value.

Superoxide is formed both as a consequence of ionization of water and also by activated immune cells, which secrete both superoxide and nitric oxide. Breakdown products of superoxide and nitric oxide have been shown to be highly genotoxic (19,32). Furthermore,

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superoxide and nitric oxide can react to form peroxynitrite, which has been shown to be highly recombinogenic (33). A reduction in the levels of superoxide might therefore reduce the levels of DNA damage. To explore this possibility, we tested MnSOD-PL gene therapy. We found that MnSOD-PL delivered in a concentrated organ-specific fashion significantly reduced the magnitude of radiation-induced HR in the esophagus *in vivo*. These data suggest that MnSOD-PL can exert radioprotective effects in the esophagus at the level of DNA damage, which is detectable as a reduction in radiation-induced HR in individual explanted cells. Alternative explanations include the possibility that MnSOD-PL treatment inhibits the HR process or that MnSOD expression suppresses expression of the YFP protein.

The observation that MnSOD-PL suppresses the genotoxic effects of radiation is consistent with published data on the effects of intraesophageal administration of MnSOD-PL including increased mouse survival (1), preservation of body weight and hydration (1), protection of self-renewing transplantable esophageal stem cells (6,11), decreased radiation-induced apoptosis (10), and decreased radiation-induced lipid peroxidation (9).

We also explored the potential effects of MnSOD in the pancreas (which has been studied previously) using FYDR mice (16,18,29) and in the bone marrow (which had not previously been studied for HR in FYDR mice) after thoracic irradiation. HR was also suppressed in the pancreas of mice receiving thoracic irradiation after intraesophageal administration of MnSOD-PL. MnSOD administered to the esophagus might lead to a reduction in HR in the pancreas as a result of the ability of MnSOD-PL to reduce the levels of radiation-induced cytokines that might otherwise induce HR (3). Since radiation did not produce a statistically significant increase in the level of HR in the pancreas, the apparent suppression of HR by administration of MnSOD-PL is inconclusive. There was no detectable increase in HR in the bone marrow of the same FYDR mice. The observation that relatively few bone marrow cells from the positive control FYDR-Rec were detected as $YFP^+$ suggests that expression of EYFP in bone marrow limits the detection of rare fluorescent recombinant cells. The observed variation in the spontaneous levels of recombinant fluorescent cells in different mouse tissues confirms and extend previous studies (16–18).

To further explore the potential for MnSOD to suppress HR in the bone marrow, we irradiated the femurs and tibias of other FYDR mice with 8 or 29 Gy and quantified HR 1 to 14 days after irradiation. Earlier times were chosen for bone marrow examination since hematopoietic stem cells and their progeny are significantly more radiosensitive and undergo apoptosis more rapidly after irradiation than esophageal cells (9,10). Radiation induced a detectable increase in HR at day 7 in bone marrow irradiated with 8 Gy, but no increase in recombinant fluorescent cells was apparent on day 14.

Given the very low level of EYFP expression in the bone marrow of the positive control mice, the significant increase on day 7 was unexpected.

Intravenous injection of MnSOD-PL 24 h prior to irradiation did not significantly reduce the elevation of HR in the 8 Gy-irradiated bone marrow. This may have been the result of a low concentration of MnSOD-PL reaching the marrow after i.v. injection compared to the relatively high levels achieved in the locally treated esophagus. Systemic delivery of MnSOD-PL by intravenous injection does protect mice from death after 9.5 or 1.0 Gy total-body irradiation, but uptake of plasmid in marrow was not quantified in that study (34). We administered 100 μg of plasmid DNA in both intraesophageal and i.v. procedures. The local esophageal delivery results in a detectable level of expression of the MnSOD transgene in the esophagus for 24–72 h (1). In recent studies, at 24 h after i.v. injection of MnSOD-PL, examination of all explanted mouse tissues by PT-PCR did not detect MnSOD transgene-specific sequences in marrow, only in the liver. Moreover, we did not detect epitope-tagged transgene encoded HA-

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MnSOD protein by histochemistry at 24 h in explanted marrow, only in the liver. In contrast, after local intraesophageal administration, the transgene and its HA-MnSOD product protein were detected in explanted tissue continually for 24–72 h (1). Further studies testing the effect of higher levels of local intramedullary injected MnSOD-PL on irradiated bone marrow may help to evaluate the role of marrow levels of transgene and protein in radioprotection.

Radiation was delivered locally to the esophagus and marrow of FYDR mice at a dose permitting survival of some stem cells (35) (29 Gy for esophagus, 8 Gy for marrow) and induced an increase in HR. A marrow dose of 29 Gy was also given, which was expected to leave no surviving hematopoietic progenitors. We did not observe any induction of HR by an increase in the frequency of $YFP^+$ cells at this toxic dose. This is in contrast to the results for the less toxic dose of 8 Gy. It is not surprising that a recent publication (36) reported no esophageal stem cell killing after a lower, fractionated radiation dose. The present data provide useful information on the effects of radiation on the bone marrow of FYDR mice. In particular, these studies suggest that FYDR mice could be useful for evaluating the potential benefits on hematopoietic stem cells of systemic i.v. and/or transdermal delivery of new small-molecule mitochondrially targeted radioprotectors and radiation damage mitigators.

Large-scale DNA sequence rearrangements that result from HR are known to contribute to cancer and aging, and HR can be induced by DNA damage caused by radiation. Methods for suppressing radiation-induced HR would therefore be of value. Here we explored the potential for gene therapy to protect against radiation-induced HR and showed that MnSOD-PL suppressed radiation-induced HR in both the esophagus and the pancreas. In contrast, suppression of HR was not observed in the bone marrow, possibly as a result of limited delivery to target cells. While prolonged overexpression of the MnSOD transgene has been shown to reduce levels of other antioxidant proteins such as catalase (37), acute overexpression in the gene therapy model does not. The demonstration that MnSOD-PL suppresses HR *in vivo* shows that a reduction in the levels of superoxide helps to prevent radiation-induced genotoxicity and provides evidence of an additional benefit of MnSOD-PL therapy.

## Acknowledgments

This work was supported primarily by NIH Grant R01-CA83876-8 and U19A1068021 from NIAID. DMW was supported by T32-ES07020. We also acknowledge support from DOE DE-FG01-04ER04 and NIH P01-CA26735.

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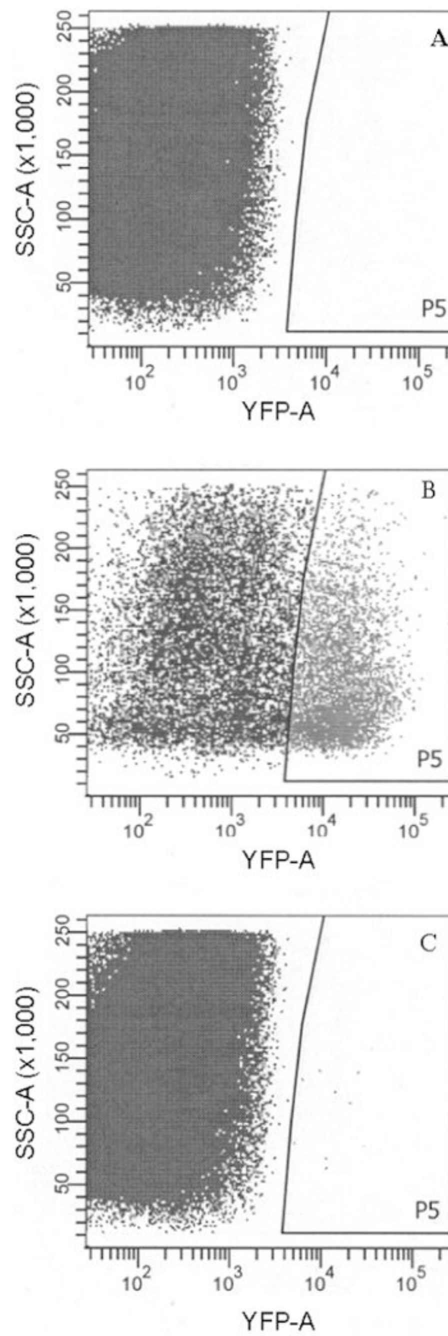


**FIG. 1.**

Flow cytometry analysis of single cells from explanted esophagus of a representative nonirradiated (panel A) C57BL/6NHsd, (panel B) FYDR-rec, and (panel C) FYDR mouse. SSC-A = Side scatter granularity.

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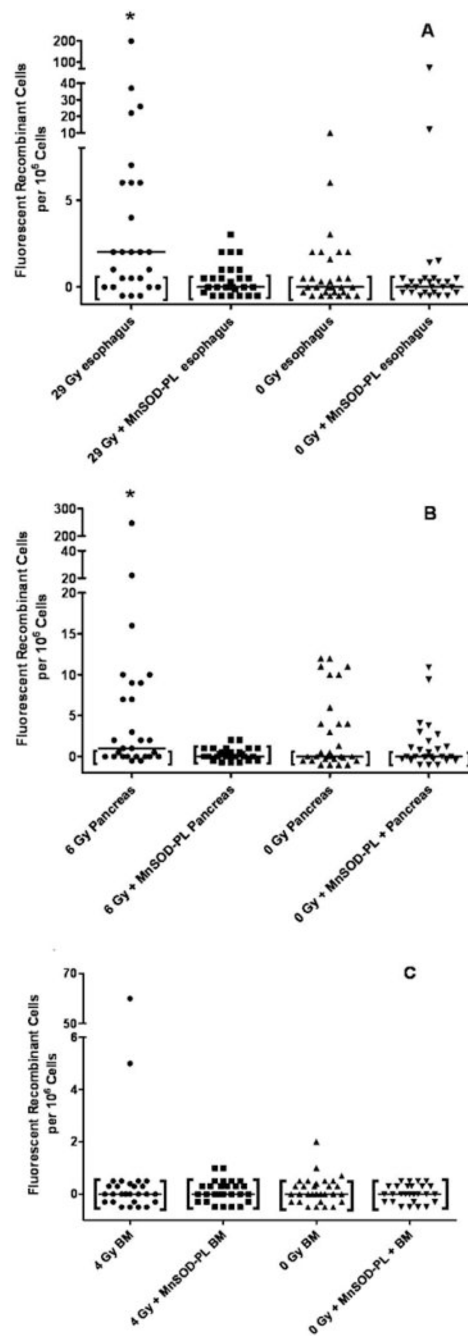


**FIG. 2.**

Effect of MnSOD-PL on homologous recombination in the esophagus (panel A), pancreas (panel B) and bone marrow (panel C) of unirradiated FYDR mice or FYDR mice irradiated with 29 Gy. The results are shown as a scatter plot with the median indicated by a horizontal line. All symbols within the brackets indicate 0 YFP cells per $10^6$ cells. In panel A, * indicates a significant difference from the other three groups. In panel B, * indicates a significant difference compared to MnSOD-PL + 6 Gy.

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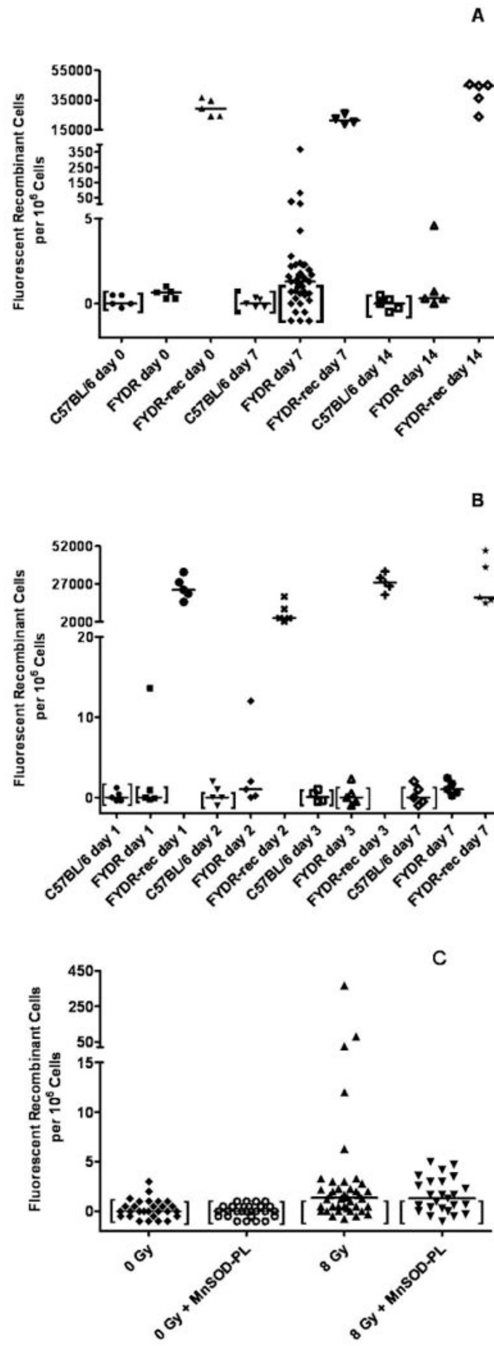


**FIG. 3.**

Homologous recombination in FYDR mouse bone marrow after 8 Gy. All symbols enclosed in the brackets are 0 YFP$^+$ cells per 10$^6$ bone marrow cells. Seven days after 8 Gy, FYDR mice showed a significant increase in the number of YFP$^+$ cells in the marrow of the irradiated compared to the control nonirradiated leg. Each symbol in panels A and B represents ≥5 mice/group. In panel C, there were 25 mice/group. Systemic i.v. administration of MnSOD-PL did not significantly decrease the radiation-induced increase in YFP$^+$ cells.

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## TABLE 1

### Comparison of the Number of YFP$^+$ Cells at Day 7 Postirradiation in Esophagus, Pancreas and Bone Marrow from FYDR Mice

| Tissue | Treatment | Number of mice | Number of YFP$^+$ cells per $10^6$ cells: median (IQR[a]) | $P$[b] |
|---|---|---|---|---|
| Esophagus | 29 Gy | 27 | 2.2 (0–6.8) | 0.0008 (compared to MnSOD + 29 Gy) |
| | | | | 0.0026 (compared to 0 Gy) |
| | | | | 0.0004 (compared to MnSOD + 0y) |
| | MnSOD + 29 Gy | 27 | 0 (0–1.1) | |
| | 0 Gy | 30 | 0 (0–1.6) | |
| | MnSOD + 0 Gy | 27 | 0 (0–0) | |
| Pancreas | 29 Gy | 27 | 1.4 (0–9.0) | 0.0039 (compared to MnSOD + 29 Gy group) |
| | MnSOD + 29 Gy | 27 | 0 (0–1.2) | |
| | 0 Gy | 30 | 0 (0–4.1) | |
| | MnSOD + 0 Gy | 27 | 0 (0–1.9) | |
| Bone marrow | 29 Gy | 27 | 0 (0–0) | |
| | MnSOD + 29 Gy | 27 | 0 (0–0) | |
| | 0 Gy | 30 | 0 (0–0) | |
| | MnSOD + 0 Gy | 27 | 0 (0–0) | |

[a] Interquartile range: the range from the first quartile to the third quartile.

[b] Two-sided Mann-Whitney *U* test. *P* values are shown only for statistically significant differences.

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**TABLE 2**

**Homologous Recombination in Irradiated Bone Marrow from C57BL/6NHsd, FYDR and FYDR-rec Mice**

**A.**

| | **Days after 8 Gy irradiation** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | | **7** | | **14** | |
| **Mouse strain** | **No. YFP cells/$10^6$ cells** | **Percentage of YFP cells** | **No. YFP cells/$10^6$ cells** | **Percentage of YFP cells** | **No. YFP cells/$10^6$ cells** | **Percentage of YFP cells** |
| C57BL/6NHsd | 0.01 ± 0.01 | 0.0001 ± 0.0001 | 0.1 ± 0.1 | 0.0001 ± 0.0001 | 0.1 ± 0.1 | 0.0001 ± 0.0001 |
| FYDR | 0.6 ± 0.1 | 0.0001 ± 0.0001 | 3.1 ± 2.3 | 0.0003 ± 0.0002 | 1.2 ± 0.9 | 0.0002 ± 0.0001 |
| FYDR-rec | 29624 ± 2602 | 3.0 ± 0.3 | 21706 ± 1518 | 2.3 ± 0.1 | 42082 ± 1830 | 4.2 ± 0.2 |

**B.**

| | Days after 29 Gy irradiation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 1 | | 2 | | 3 | | 7 | |
| Mouse strain | No. YFP cells/$10^6$ cells | Percentage of YFP cells | No. YFP cells/$10^6$ cells | Percentage of YFP cells | No. YFP cells/$10^6$ cells | Percentage of YFP cells | No. YFP cells/ $10^6$ cells | Percentage of YFP cells | No. YFP cells/$10^6$ cells | Percentage of YFP cells |
| C57BL/6NHsd | 0.01 ± 0.01 | 0.0001 ± 0.0001 | 0.1 ± 0.1 | 0.0001 ± 0.0001 | 1.6 ± 0.8 | 0.0002 ± 0.0001 | 1.5 ± 1.0 | 0.0002 ± 0.0001 | 1.9 ± 0.7 | 0.0015 ± 0.0012 |
| FYDR | 0.6 ± 0.1 0 | .0001 ± 0.0001 | 3.0 ± 2.7 | 0.0001 ± 0.0001 | 3.1 ± 2.3 | 0.0001 ± 0.0001 | 0.3 ± 0.3 | 0.0001 ± 0.0001 | 1.2 ± 0.2 | 0.0001 ± 0.0001 |
| FYDR-rec | 29624 ± 2602 | 3.0 ± 0.3 | 23088 ± 1871 | 2.3 ± 0.2 | 9010 ± 2690 | 0.9 ± 0.3 | 27002 ± 2108 | 2.7 ± 0.2 | 27186 ± 6905 | 2.7 ± 0.7 |

*Note.* The numbers shown are means ± SE.

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## TABLE 3

**Statistical Analysis of Effects of MnSOD-PL on Homologous Recombination in Irradiated FYDR Mouse Bone Marrow**

| Group | Number of mice | Median number of YFP$^+$ cells per $10^6$ cells and IQR[a] (in parentheses) | P[b] |
|---|---|---|---|
| 0 Gy | 35 | 0 (0–0.3) | |
| MnSOD-PL + 0 Gy | 27 | 0 (0–0) | 0.017 compared to 0 Gy |
| 8 Gy | 40 | 0.5 (0.2–1.5) | <0.0001 compared to 0 Gy |
| MnSOD-PL + 8 Gy | 25 | 0.7 (0.3–3) | <0.0001 compared to 0 Gy |

[a] Interquartile range: the range from the first quartile to the third quartile.

[b] Two-sided Mann-Whitney *U* test. *P* values are shown only for statistically significant differences.