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SYSTEMIC POLYETHELENE GLYCOL-MODIFIED (PEGYLATED) - SUPEROXIDE DISMUTASE AND -CATALASE MIXTURE ATTENUATES RADIATION PULMONARY FIBROSIS IN THE C57BL/6 MOUSE

M. Machtay, M.D.^{2,4}, A. Scherpereel, M.D.¹, J. Santiago, M.D.-Ph.D.¹, J. Lee, MD¹, J. McDonough, Ph.D.², P Kinniry, M.D.¹, E Arguiri, B.S.¹, V.V. Shuvaev, MD-Ph.D¹, J. Sun, MD.¹, K. Cengel, MD.-Ph.D.², C.C. Solomides, M.D.³, and M. Christofidou-Solomidou, Ph.D.¹

¹ Departments of Medicine, Pulmonary Allergy and Critical Care Division and

² Radiation Oncology, University of Pennsylvania Medical Center, Philadelphia PA 19104,

³ Department of Pathology, and Temple University Hospital, Philadelphia PA 19140, USA,

⁴ Department of Radiation Oncology, Thomas Jefferson University Medical Center, Philadelphia PA 19107

Abstract

Purpose—Since oxidative injury is implicated in radiation-induced tissue damage to the lung, we studied systemically administered polyethylene glycol (PEG-ylated) antioxidant enzymes (AOEs) as pulmonary radioprotectors in mice.

Methods and Materials—C57Bl Mice received 13.5 Gy single-dose irradiation to the thorax. One cohort also received 100µg of a 1:1 mixture of PEG-AOE's {PEG-Catalase and PEG-Superoxide Dismutase (SOD)} intravenously, pre-irradiation and subgroups were evaluated at variable time-points for inflammation and fibrosis. Potential for AOE tumor protection was studied by thoracic irradiation of mice with Lewis Lung Carcinoma.

Results—At 48 hours post-irradiation, control irradiated mice had marked elevations of tissue p21, Bax and TGF-β1 in lungs, not seen in irradiated, PEG-AOE-treated mice. TUNEL staining of lung sections was performed at just one time-point (24 hours post-irradiation) and revealed a decrease in apoptotic cells with AOE treatment. At four months post-irradiation, these mice had significantly increased pulmonary fibrosis as measured by hydroxyproline content. Mice treated with PEG-AOE prior to irradiation had 4-month hydroxyproline levels that were similar to that of unirradiated controls (p=0.28). This corresponded to less pulmonary fibrosis as visualized histologically when compared with mice irradiated without AOE's. PEG-AOEs did not prevent post-irradiation pulmonary inflammation or lung cancer response to irradiation.

Conclusions—A mixture of PEG-SOD and PEG-CAT successfully diminished radiation pulmonary fibrosis in mice. There was also a corresponding effect on several early biomarkers of

Address Correspondence to: Melpo Christofidou-Solomidou, Ph.D: ¹Department of Medicine, Pulmonary, Allergy, and Critical Care Division, University of Pennsylvania, 421 Curie Blvd., BRB II/III, Philadelphia, PA 19104. Phone: (215)-573-9917; Fax# (215) 573-4469; e-mail: melpo@mail.med.upenn.edu.

CONFLICT OF INTEREST NOTIFICATION

The co-authors of this manuscript state that there is no conflict of interest.

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lung injury and decreased apoptosis. There were no significant effects on acute pneumonitis or tumor protection.

Keywords

Antioxidant enzymes; radiation pneumonopathy; lung fibrosis; TGF-beta; superoxide dismutase; catalase; PEG; inflammation; thoracic radiation; apoptosis

Abbreviations

AOE= Antioxidant Enzymes; BAL F= Bronchoalveolar lavage fluid; CAT=Catalase; H&E = hematoxylin and eosin; HPLC=High performance liquid chromatography; LLC= Lewis Lung Carcinoma; MDA= Malondialdehyde; PEG= Polyethylene glycol ; PMN = Polymorphonuclear leukocyte; SOD= Superoxide Dismutase; WBC = White blood cells; XO= xanthine/xanthine oxidase; XRT: X-ray Treatment

INTRODUCTION

The utility of thoracic radiotherapy is limited by the high radiosensitivity of the normal lung parenchyma (1,2). Clinically significant radiation lung injury occurs in up to 30% of patients irradiated for lung cancer (3) and about 10–15% of other thoracic oncology patients (4). A far greater proportion of patients will have subclinical adverse effects of radiation on the lung, identifiable by imaging and/or physiologic testing (5).

Two types of radiation lung injury have been described (1). Acute radiation pneumonopathy (pneumonitis) can occur several weeks to six months post-irradiation. If a large volume of lung has been affected, this phase can be life-threatening (6). In the second type of radiation-induced lung injury, months to years after irradiation, the lung tissue enters the “fibrotic” phase, in which the number of inflammatory cells decrease and a marked thickening of alveolar walls -- due to collagenous deposition -- occurs. This later process has also been modeled in animals; the C57/B6 strain of mice seems especially susceptible to this fibrotic reaction (7).

Manipulating the response to radiation at the cellular level is a major focus of academic radiation oncology. Highly reactive, injurious compounds, known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are induced by radiation (8). Recent studies, reviewed by Robbins & Zhao (9) suggest a role for chronic oxidative stress in radiation-induced late effects; it is widely recognized that irradiation is associated with both a rapid and a chronic increase in reactive oxygen and reactive nitrogen species (ROS/RNS) production. These damaging chemicals may be targets for molecular inhibition of radiation injury and thus radioprotection (10). It is hypothesized that radiation-injured cells within the lung release numerous pro-fibrotic cytokines, as reviewed by Rubin (11), one of which is TGF-beta (12, 13). However, it is likely that numerous other molecular events including activation of various genes contribute to the complex pathogenesis of radiation pneumonopathy.

Currently the only means to avoid life-threatening or fatal radiation pneumonopathy is to strictly minimize the amount of lung exposed to dangerous radiation levels. Given the limitations inherent in dosimetry, however, a safe and effective biologic radioprotector would be extremely useful. Preclinical data suggest that antioxidant molecules and/or enzymes might offer protection of the lung (14–16). We explored the potential pulmonary radioprotective efficacy of specially conjugated (PEGylated) antioxidant enzymes (AOEs) -- superoxide dismutase and catalase -- when given systemically immediately prior to radiation treatment in a well-established preclinical murine model (C57Bl/6) of radiation lung injury. These compounds have demonstrated efficacy in other models of lung injury such as sepsis models

(17). This study further investigated the kinetics of molecular biomarker expression (Bax, p21 and TGF- β 1) in irradiated lungs and evaluated their usefulness in measuring effectiveness of AOE therapy.

MATERIALS AND METHODS

Reagents

The following reagents were used in the study: PEG-catalase and PEG-SOD (Cu/Zn-SOD from bovine erythrocytes) from Sigma (St. Louis, MO), Bradford BioRad protein microassay kit from (BioRad Laboratories, Hercules, CA).

Animals

Our studies used female C57Bl/6 mice, a strain well characterized in the field of pulmonary radioprotection (18). Mice were obtained from Charles River (Wilmington, MA). Mice were irradiated at the age of approximately 6–8 weeks in groups of 8 mice per irradiation round. Ten mice per group were used in all experiments unless otherwise stated in text. Prior to and after irradiation, they were housed in standard cages and were fed a normal chow diet. All procedures on animals were performed in accordance with the ethical treatment of the animals being studied and with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

Radiation and Drug Administration Procedures

The radiation source was a 250kVp orthovoltage machine, with a current of 13mA. A customized jig was created that allowed the simultaneous irradiation of six to eight mice simultaneously and homogeneously; lead shielding was used over the animals' head/neck and abdomen/pelvis, exposing only the entire thorax to irradiation. The midplane dose (not corrected for air/lung/tissue inhomogeneity) was a single fraction of 13.5 Gy (8-minute radiation session). Dosimetric analysis, including thermoluminescent dosimeters (TLDs) was performed by one of the coauthors (JM) and confirmed that dose heterogeneity in the animals' lungs was within 15%.

Approximately 15 minutes before irradiation, mice were anesthetized with ketamine/xylazine (70/7 mg/kg). Cohorts of mice were assigned to receive either saline or a drug mixture of 100 μ g Polyethylene glycol conjugated Superoxide Dismutase (PEG-SOD) and 100 μ g PEG conjugated Catalase (PEG-CAT) via tail vein injection in a 200 μ l bolus, approximately 10–15 minutes prior to irradiation (immediately after anesthetization). After the completion of irradiation, mice were given 1mL of saline injection subcutaneously in order to prevent post-radiation dehydration and/or other toxicities from radiation exposure.

Confirmation of AOE activity in murine plasma and lung tissues

In a separate cohort of mice, SOD and catalase enzymatic activity levels in plasma and lung homogenates of saline-perfused, blood-free animals were measured at 15 minutes post-injection (which corresponds to the timing of irradiation) as described by White et.al (19). These levels were compared to plasma and lung tissue levels within control animals not injected with AOE's. The experiments were performed twice (n=3 per group) and all measurements were run in triplicates.

SOD activity assay—Activity of SOD was measured using ferricytochrome c assay as previously described (20). Cytochrome c assay uses xanthine and xanthine oxidase (XO) as a source of superoxide radicals and cytochrome c as the indicating scavenger of the radical.

Catalase activity—Catalase activity was measured directly by the kinetics of 5 mM H₂O₂ degradation at 242 nm. The slope of the curve $\Delta A/\text{min}$ was estimated using initial linear fragment and catalase activity in plasma was calculated as follows: catalase activity, U/ml = $23.0(\Delta A/\text{min})/\text{ml}$ of plasma.

Apoptosis detection in lung tissues by TUNEL staining

In order to identify the extent of potential cell apoptosis/necrosis as a consequence of DNA damage, we performed terminal transferase dUTP nick-end-labeling (TUNEL)-staining on lung sections from mice injected with PEG-AOEs and irradiated as described above. Lungs were excised at 24 hours post-XRT and processed for paraffin embedding. Lung sections were deparaffinized and hydrated before blocking of endogenous hydrogen peroxide with hydrogen peroxide-methanol. TUNEL staining was performed using an ApopTag kit obtained from Oncor (Gaithersburg, MD), as previously described (21).

Mouse Survival, Tissue Harvesting and Evaluation of Lung Injury

Mouse survival was recorded twice a week post XRT and the experiment was terminated after 4 months, approximately corresponding to the earliest time point when radiation-induced fibrosis was detectable. Separate animals were used for short-term experiments intended for molecular studies or determination of inflammatory parameters (24, 48, 72 hours or 1, 2, 3 weeks).

After animal euthanization via anesthetization followed by cervical dislocation, lungs were harvested, inspected, photographed and lavaged for BAL protein, WBC and PMN determination or processed for histology. Sections were stained with hematoxylin and eosin (H&E), and examined by light microscopy or with Mason's trichrome blue (MTB) stain for evaluation of collagen deposition/fibrosis. Assessment of BAL injury parameters was performed as previously described (7).

Quantitative Assessment of Fibrosis-Quantification of Lung Hydroxyproline Content

Whole collagen content of mouse lung was evaluated by determining hydroxyproline content. Briefly, after recovery of the bronchoalveolar lavage fluid (BALF), all lung lobes were removed, weighed and cut into sections (1 mm thick). Then, the dried lung samples were hydrolyzed with 2 mL of 6 N HCl at 120°C for 16 h in sealed glass tubes. The amount of hydroxyproline in the hydrolysate was measured according to Woessner et.al.(22). Commercial hydroxyproline (hydroxy-L-proline, Sigma) was used to establish a standard curve. The data is expressed as μg hydroxyproline/whole lung.

Analysis of Malignant Tumor Radioprotection

In order to test for potential "tumor protection" by AOE administration, a cohort of mice was injected iv with 2 million Lewis Lung carcinoma cells (LLC cells), a murine lung carcinoma cell line (23). Multiple metastatic lung tumors were established within 2 weeks post injection as judged by parallel animals that were sacrificed and evaluated histologically. At this time, mice were injected iv with 100ug of PEG-AOE mixture and given a single dose XRT to the thorax (13.5 Gy). As controls, we used a) animals with tumors but without PEG-AOE treatment that were given XRT, or b) animals with tumors that were not given XRT. Mice were allowed to live 6 days post radiation, lungs were excised and weighed and tumor burden evaluated.

Oxidative Modification of Irradiated Lungs

Pulmonary oxidative stress was detected by evaluation of tissue malondialdehyde (MDA), a measure in vivo lipid peroxidation in lung tissues. This was performed using a commercially available kit (Oxis International Inc., Portland Oregon).

RNA Preparation

After sacrifice at selected time-points, lungs were immediately placed in 4M guanidine isothiocyanate, 0.5% N-laurylsarcosine, 25mM sodium citrate, and 0.1M β -mercaptoethanol solution and homogenized. Total lung RNA was isolated using a modified one step method of acid guanidinium-thiocyanate phenol-chloroform extraction followed by removal of contaminating genomic DNA by DNase I treatment. Only RNA with a 260/280 ratio of > 1.7 was used. To check for genomic DNA contamination, 2 mg of total RNA was used as a template in a PCR reaction with the primers for intronic sequences of the mouse PECAM-1 gene. No visible PCR product in total RNA sample was detected after 35 cycles, together with a positive control using as low as 500 pg of genomic DNA as a template in the PCR reaction.

Semi-quantitative RT-PCR – Confirmation of Selected Genes

Semi-quantitative analysis of mRNA expression was performed as described (24) to confirm and/or evaluate the differential expression of p21, Bax and TGF- β 1 genes at 1,2,7,14 and 21 days, as early biomarkers of the irradiation response and monitor their modulation by the AOE therapy. TGF- β is a key cytokine associated with the repair of normal tissue injury and it has been implicated as a key cytokine in the induction of fibrosis in many organs, including the lung (13). Since the formation of ROS following irradiation is thought to be a major determinant of cellular damage and apoptosis (25) in normal tissues, in addition to evaluating TGF- β gene expression, we evaluated the apoptosis-related proteins p21 and bax.

Two mg of total RNA were reverse transcribed to cDNA using Oligo(dT)15 primer (Promega, Madison, WI) and powerscript reverse transcriptase (Clontech). Synthesized cDNA was then submitted to real-time PCR using either the LightCycler System (Roche Molecular Biochemicals) or Smart Cyclers. The amount of cDNA was normalized using beta-actin levels. A minimum of 3 samples from control and irradiated lungs were pooled and analyzed in quadruplicate. The relative expression level based on cycle number was compared between groups.

Statistical Analysis

Statistical differences among groups were determined using one-way analysis of variance (ANOVA). When statistically significant differences were found ($p < 0.05$) individual comparisons were made using the Bonferroni/Dunn test (Statview 4.0). Kaplan-Meier survival curves were analyzed with the Mantel-Cox log-rank test. Results are expressed as mean $\pm$ SEM.

RESULTS

Effect of PEG-SOD and PEG-Catalase mixture on acute radiation pneumonitis

C57/bl/6 mice develop radiation pneumonitis as early as 3 weeks post single fraction XRT (13.5GY) (Fig. 1). Systemic PEG-AOE (100 μ g/mouse for each PEG-ylated AOE) given i.v. at the time of radiation did not alleviate lung injury or inflammation as judged by BAL protein levels or BAL neutrophils respectively at any time-point investigated (1,2,3,7,14,21 and 120 days post XRT).

Effect of AOE's on oxidative modification and apoptosis in irradiated lung tissues

Malondialdehyde (MDA) is a product of lipid peroxidation, which in turn is a result of free radical interactions with lipid molecules in the cell. MDA is thus a potential marker of the amount of radiation-induced oxidative injury to the cell (26). MDA in mouse lung homogenates increases significantly within just 2 days post radiation and is sustained for several days (Fig. 2A). Mice that were given systemic PEG-AOE at 100 μ g/mouse of each AOE at the time of thoracic irradiation indicated a sustained low level of lipid peroxidation as compared to

irradiated, non-treated counterparts that showed significantly higher levels of MDA at all time-points tested ($p=0.003$ for 2,7,14,21 days post XRT) (Fig. 2a). Apoptosis in irradiated lung tissues, as evidenced by TUNEL staining (2C) at 24 hours post XRT was inhibited by AOE treatment although admittedly evaluation was only performed at a single and relatively late time-point post-irradiation and may not reflect the true extent of apoptosis inhibition. Semiquantitative assessment of apoptosis (TUNEL-positive cells/high power field) represented graphically (fig.2B) indicated a 14-fold decrease of apoptosis with enzyme treatment.

Plasma and Lung Tissue measurements of AOE's

The mean value for SOD activity in mouse plasma 15 minutes after exogenous iv PEG-SOD injection was 158.7 ± 7.23 U/ml. This was significantly higher than the value found in a control group of mice (17.8 ± 4.1 U/ml), representing a 9-fold increase over circulating, endogenous SOD activity levels. Similarly, the mean value for catalase activity in plasma 15 minutes after exogenous PEG-catalase injection was 1.2 ± 0.01 kU/ml, representing a 52-fold increase over circulating endogenous levels, while the catalase activity in a control group of mice was negligible (0.024 ± 0.004). Contrary to elevated plasma concentrations in the circulation, lung tissue activity levels of the enzymes in PEG-AOE-injected mice as compared to saline-injected controls, remain unchanged ($1,096.1 \pm 49.2$ U/ml vs. 960.1 ± 94.7 U/ml for SOD) and (131 ± 22 U/ml vs. 134 ± 11 U/ml for catalase). We used for this analysis, tissues from 4 animals per experimental group.

Effect of PEG-AOE treatment on early activation of apoptotic and cell cycle progression inhibitor genes

We showed (Figure 3A) that gene expression for both Bax and p21 is significantly ($p < 0.0001$) increased over baseline controls within days and up to several weeks post a single fraction thoracic XRT. To test whether a single treatment of systemic PEG-AOEs given at the time of irradiation might interfere with the cascade of intracellular events leading up to cell death, we evaluated lungs for Bax and p21 mRNA levels (Figure 3B) after 48 hours of irradiation and treatment, the time point of the most robust upregulation of gene expression of both genes (Bax and p21). While radiation caused a 13 and 6-fold increase of both p21 and Bax genes respectively by 48 hours post XRT, the AOE treatment significantly ($p = 0.0001$) prevented the increase and kept the mRNA levels at baseline values.

Effect of Systemic PEG-AOE treatment on TGF- β 1 gene expression in irradiated lungs

Kinetics of TGF- β 1 gene expression levels in irradiated mouse lungs following a single-fraction radiation treatment was examined using mRNA from lung homogenates. TGF- β 1 levels (Means $\pm$ Standard Deviation) were 1 ± 0.31 , 2.58 ± 1.11 , 3.46 ± 0.06 , 3.15 ± 0.82 , 2.16 ± 0.93 , $\pm 1.39 \pm 0.02$ and 1.70 ± 0.44 for control, 24 hr, 48 hr, 72 hr, 1wk, 2wk and 3 wks post XRT. Data was normalized to β -actin ($n=3$ mice per time point). We identified an early increase at 48 hours post-XRT. This is in agreement with previous work (13). A single dose PEG-AOE mixture given at the time of XRT, prevented the early, radiation-associated, TGF- β 1 mRNA gene increase while the non-treated irradiated group showed a significant ($p=0.005$ from non-irradiated controls) increase in expression (1.00 ± 0.14 vs. 2.65 ± 0.19 vs. 1.01 ± 0.06 , for non-irradiated control, vs. XRT/no drug, vs. XRT+ PEG-AOE).

Effect of PEG-AOE treatment on lung fibrosis from single fraction X-ray radiation to the thorax in mice

Data on hydroxyproline content (a semi-quantitative measurement of fibrosis) at four months post-irradiation representing a meta-analysis of three separate, sequential experiments comparing non-irradiated controls versus irradiated controls versus animals irradiated with

AOE protection, indicated that irradiated controls had significantly increased hydroxyproline content as compared with non-irradiated controls ($p = 0.0076$). The hydroxyproline levels for the group of animals irradiated with AOE protection were intermediate; there was no statistically significant difference in hydroxyproline content between non-irradiated controls and animals irradiated with AOE protection ($p=0.28$), suggesting a protective effect of AOE's.. Histopathologic changes from representative animals are shown in Figure 4. The irradiated, non-treated group of lungs, show significant expansion of alveolar septa with increased mononuclear inflammatory infiltrate and increased collagen (see MTB-stained slides in Fig 4) with foci of subpleural and peribronchial fibrosis. There is also pneumocyte II hyperplasia, and many intraalveolar macrophages. The treated (PEG-AOEs) group of irradiated lungs, had less severe fibrosis.

Survival

Overall, no statistically significant survival differences were observed between irradiated, PEG-AOE-treated and non-drug treated controls ($p=0.8569$) Nevertheless, the overall survival rate at 4 months yielded a sufficient number of animals to allow the semiquantitative and quantitative analysis of the fibrotic status of the lungs in each experiment.

Effect of PEG-AOE treatment on a tumor model response to irradiation

We tested the effect of PEG-AOE treatment on established lung tumors (LLC cells) in conjunction with radiation therapy. The tumor burden normally causes a near 5-fold increase in lung weight within just 3 weeks post-injection into the mice. Irradiation slowed tumor growth to 2.5-fold and 2.0 fold increase from non-irradiated control lungs with or without AOE treatment respectively. In general, irradiated lungs were smaller than non-irradiated tumor controls, weighed less (Fig. 5) and had fewer visible tumor nodules ($p=0.001$ from non-irradiated tumor controls). No obvious difference in appearance or weight was observed with AOE treatment in irradiated, tumor-bearing lungs.

DISCUSSION

These studies provide strong evidence that systemically-delivered, non-targeted antioxidant drug therapy (PEG-SOD and PEG-CAT enzymes) can decrease some of the manifestations of radiation pneumonopathy. Specifically, several early molecular events were diminished and the important "late" effect of fibrosis was also reduced. Interestingly, however, the intermediate phase of radiation lung injury – inflammation or pneumonitis – was not affected at all. This supports the hypothesis that early/intermediate radiation pneumonitis and long-term radiation fibrosis may be considered as two separate events, perhaps reflecting damage to different target cells within the lung. It is even possible that radiation pneumonopathy might come to be viewed as a three-phase set of events: Phase I representing hyperacute molecular changes; Phase II representing inflammatory response; and Phase III representing late atrophy and fibrosis.

We elected to use a combination of two enzymes (catalase and SOD) rather than each AOE alone since the two enzymes complement each other; the harmful end product (H_2O_2) of one enzyme (SOD) is decomposed to harmless components by the other enzyme (catalase). In previous study, they were shown to work effectively in protecting rabbit lungs and rat lungs from hyperoxic insult (19,27). Also importantly, we opted to utilize Pegylated forms of these AOE's, because the covalent binding of PEG to AOEs extends their plasma half-lives (28, 29). Such PEG-modification of catalase and SOD blocks their clearance by the kidneys and decreases their resistance to enzymatic degradation (28).

Our study supports the concept that the late effect of lung irradiation (radiation fibrosis) results from a cascade of cytokines that in fact begins relatively quickly after irradiation (11). We

demonstrated that TGF- β 1 mRNA levels surge soon after irradiation and that this elevation is reversible with radioprotective AOE's. Our results also indicated that a single, systemic, early antioxidant intervention, at the time of radiation inhibits downstream oxidative events such as lipid peroxidation in lung tissues. The AOE's downregulated cell cycle progression (p21) and apoptotic (Bax) signals during the early phase of radiation-induced oxidative stress and cell damage/survival. Bax protein upregulation appears to be a particularly early molecular event after irradiation of cells that are sensitive to irradiation (30). A study by Bouvard and coworkers (31) investigated early increases (up to 6 hours post XRT) and confirmed an increase in both Bax and p21, but our study demonstrated prolonged (days to weeks) changes in expression of these genes.

We demonstrated high plasma activity of catalase and SOD, confirming previously reported data (32). The levels of these enzymes in blood-free lung tissues were low, since PEGylated AOE's does not specifically target any one cell type (33). The lung receives 100% of the cardiac output and is extremely highly vascularized; thus we hypothesize that high plasma levels of these enzymes at the time of irradiation are sufficient to protect the lung parenchyma. Intravenous injection of PEG-ylated SOD and catalase increases activity in animal lungs (34) (19); this can last for over 24 hours (35) (36). The successful pulmonary radioprotection by PEG-AOE that we have shown is a very interesting observation, considering that we demonstrated that PEG-AOE's remain intravascular. It is generally believed that effective radioprotectors must localize to the nuclei of the target cells. Our study implies that radioprotection may be achievable by another mechanism. Specifically, we hypothesize that some of the radiation damage that occurs to normal cells in the lung results from free radicals produced by inflammatory cells located intravascularly and/or marginally along pulmonary vessels. Intravascular radioprotectors such as PEG-AOE's may thus scavenge these free radicals and prevent them from subsequently injuring adjacent lung cells. This would be a topic for additional molecular free radical studies. Additionally, our overall results provide strong indirect evidence that the AOE's reach their putative target(s). Specifically, we showed modulation of mRNA levels of apoptosis and cell-cycle related genes such as Bax and p21, suggesting that the AOE's indeed reached intracellular targets. Additionally, our findings of a several-day decrease of lung tissue lipid peroxidation post-XRT in PEG-AOE-treated animals also suggests that the effects are long-lasting and that AOE activity is reaching the target tissue of interest. TUNEL positivity of irradiated lungs also confirmed decreased apoptosis in the AOE + XRT cohort.

Although we demonstrated efficacy of PEG-AOE radioprotection as indicated by histological evaluation and quantitative lung hydroxyproline content, our studies may be critiqued for not measuring "clinical" endpoints such as breathing rate, lung compliance and/or animal survival. Logistically, these are relatively difficult endpoints to study. It will be important to examine these endpoints in subsequent trials. However, we note that in a preclinical study of a rat model using the successful radioprotector Amifostine, decreased lung hydroxyproline levels translated into improved breathing rates (16). We did assess survival, which was not improved with AOE's. It is possible that a survival benefit might have become evident with a larger cohort of animals followed for a longer time period. However, survival in the intermediate term after high dose radiation to the murine chest probably depends not only on late pulmonary fibrosis, but also on other events, most notably acute/subacute radiation pneumonitis, which was not influenced by AOE's. In humans, toxicity from acute pneumonitis can be ameliorated with corticosteroids, whereas there is no treatment to minimize the morbidity and mortality from late fibrosis.

We are not the first researchers to utilize AOE therapy to prevent radiation pneumonopathy. Epperly et al. showed that SOD delivered by gene therapy fashion (plasmid liposome intratracheal delivery) was an effective radioprotector (37). An earlier study by Gray and Stull

(38) evaluated radioprotection (survival) following total body radiation in mice by PEG-AOEs given iv. They concluded that PEG-SOD and PEG-Catalase in combination decreased mortality rates, while PEG-albumin or PEG-heat-denatured-catalase were not protective. To our knowledge, however, our study is the first to show that systemic, intravenous administration of modified AOEs can exert effective lung-specific.

In our experiments, we utilized a single large fraction of radiation therapy to the entire thorax. While this form of irradiation is rarely used today in clinical radiation oncology, there is some recently increased interest in large-fraction “hypofractionated” radiotherapy studies using stereotactic techniques (41). Certainly one reason for selecting a single dose of 13.5 Gy is logistical, including less stress upon the animals. Nonetheless, we believe that a single large fraction is scientifically well justified for our pre-clinical study based upon others’ successful preclinical investigations (39,40). (42,43). Lung tissue is highly sensitive to injury from large fractions of irradiation; radiobiological models predict that a single dose of 13.5 Gy to lung tissue is approximately the equivalent of a fractionated radiotherapy dose of at least 45 Gy, which is indeed a very clinically relevant dose commonly used for sterilization of microscopic carcinoma. We hypothesize that if our antioxidant treatment can protect animals from radiation pneumonopathy after a single large dose of radiation, it is reasonable to hypothesize that they could protect against fractionated radiotherapy. This has been demonstrated for the prototype radioprotector, Amifostine (16). Now that we have demonstrated radioprotection after single-dose irradiation, it will be necessary to conduct fractionated radiotherapy studies with these or similar study drug compounds in the future.

A major concern of any radioprotection study is that the protective agent(s) might theoretically protect the tumor as well, making it useless for further development. While work by Oberley et al indicated that increased AOE levels in malignant cells is associated with a reduction in tumor cell growth (45,46) other groups suggested that increased tumor cell levels of SOD are associated with cancer cell survival (47,48). This concern has prompted methods of delivering SOD specifically to normal tissues, such as gene therapy (43) (49). Since the formulation of AOEs used in our current study reported here is not specific to any one particular cell type, we assessed for the possibility of tumor protection in vitro and in vivo studies, with reassuring results. Clinical studies of Amifostine have shown no evidence of tumor protection in the treatment of thoracic malignancies (50). Nonetheless, further preclinical studies are needed to ascertain the normal tissue selectivity of AOEs as delivered in our study.

In summary, a mixture of two long-half-life antioxidant enzymes (PEG-SOD and PEG-CAT) diminished delayed radiation lung fibrosis (but not pneumonitis) after a single large fraction of whole thorax irradiation in C57 mice. Further studies of these compounds are indicated, including fractionated radiotherapy experiments, survival cohort experiments, and additional studies to rule out significant tumor protection.

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Figure 1. Evaluation of XRT-induced pulmonary inflammation and edema following AOE treatment

Comparison of BAL with respect to lung injury (BAL protein content) and inflammation (BAL neutrophil content) from irradiated murine lungs at 0,1,2,3,7,14,21 and 120 days post XRT given no drug (blue) or systemic PEG-AOE-treatment (red). AOE treatment was 100ug PEG-Catalase+100ug PEG/SOD given i.v.

There are no significant differences in the markers of inflammation between these two groups.

Figure 2. AOE Treatment decreases oxidation and apoptosis in irradiated lungs

Panel A: MDA levels were evaluated in whole mouse lung homogenates of none-irradiated, baseline controls (N=23) at T=0, and T=2, 7, 14 and 21 days (N=4 animals minimum-13 animals maximum per time-point) after systemic administration of PEG-AOEs given at 100 $\mu\text{g}/\text{mouse}$ at the time of XRT. The data are shown as Mean $\pm$ SEM, $p=0.003$ versus baseline, non-irradiated control (*).

Panel B: Semiquantitative assessment of TUNEL positive cells from 10 high-power fields (100x objective) per experimental group.

Panel C: TUNEL positive cells (40X objective) in non-irradiated controls (A,D), irradiated, saline-injected (B,E) and irradiated PEG-AOE-injected lungs (C,F). Positivity is shown as brown pigment. Top panels shows lung alveoli and bottom panels show pulmonary blood vessels.

A **B**

Figure 3. Radiation-induced changes in apoptosis-related genes encoding Bax, and inhibitors of cell cycle progression such as p21

Panel A: Kinetics of Bax and p21 gene expression in irradiated murine lungs. Panel B: Fold change in lung gene expression 48 hours following 13.5 Gy irradiation for Bax, and p21. Mice were injected iv with a single systemic bolus of PEG-AOEs at the time of XRT. Each bar represents 3–4 replicates from pooled total RNA isolated from the lungs of 5 animals. Values shown represent Means $\pm$ SD.

*, # $p < 0.0001$ from non-irradiated control for Bax and p21 respectively.

Figure 4. Qualitative analysis of radiation pulmonary fibrosis – Systemic PEG-AOE treatment decreases radiation-induced lung fibrosis

A single dose of 13.5 Gy is sufficient to induce significant fibrosis within 4–6 months in C57/bl6 mice. Assessment of radiation-induced fibrosis is done by histological evaluation of fibrotic areas. Mice were injected iv with either saline (b,e,h) or a mixture of 100ug PEG/catalase+ 100ug PEG/SOD (c,f,i) and given a single fraction thoracic radiation of 13.5 GY and evaluated 4 months later for collagen deposition using MTB stain in high (d–f, 100X objective) or low magnification (g–i, 10X objective). Irradiated mice were compared to age-matched, non-irradiated controls (a,d,g). Characteristic morphology of each group is shown here (Hematoxylin & Eosin).

Figure 5. PEG-AOE treatment and lung tumor protection

Mice were injected iv with 2×10^6 LLC cells and lung tumors were established within 2 weeks post injection. At this time, mice were injected iv with 100ug of each antioxidant enzyme (AOE) PEG-catalase and PEG-SOD and given a single dose XRT to the thorax (13.5 Gy). Mice were allowed to live 6 additional days, and lungs were excised and weighed. (N=6 mice/group).

Panels a–d: Clinical views of excised mouse lung-heart blocks. a: control, non-injected lung, b: lung with established tumors without XRT treatment, c: Lung with established tumors following XRT treatment and d: Lung with established tumors following XRT and AOE treatment. **Panels e–h:** H&E evaluations of lung sections from the corresponding lungs shown in a–d. **Panel I:** Lung weight evaluations as compared to baseline controls. The data in Panel I are shown as Mean+SEM, *P=0.001 from non-irradiated tumor controls. Asterisks mark tumor foci.