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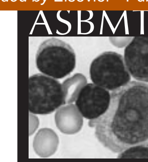

Magnesium superoxide dismutase (MnSOD) plasmid/liposome pulmonary radioprotective gene therapy: modulation of irradiation-induced mRNA for IL-1, TNF-α, and TGF-β correlates with delay of organizing alveolitis/fibrosis

Michael W. Epperly,[1] Elizabeth L. Travis,[2] Christine Sikora,[1] Joel S. Greenberger[1]

[1]Department of Radiation Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania and [2]Department of Radiobiology, Anderson Hospital, Houston, Texas

Offprint requests: Joel S. Greenberger, M.D., Professor and Chairman, Department of Radiation Oncology, University of Pittsburgh Medical Center, 200 Lothrop Street, Suite B346-PUH, Pittsburgh, PA 15213

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ABSTRACT

Radiation pneumonitis remains a critical dose-limiting toxicity of total body irradiation (TBI) for use in bone marrow transplantation. The acute and chronic phases of radiation damage in the mouse lung have been shown to correlate with mouse strain genotype and are dependent on fraction size, total dose, and total lung volume. Our prior studies demonstrated effective prevention of irradiation-induced lung damage and improved survival in C57BL/6J mice by MnSOD plasmid/liposome gene therapy. In the present studies, we investigated the kinetics of irradiation-induced upregulation of mRNA for acute phase cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α, and fibrosis-associated transforming growth factor (TGF)-ß and isoforms (TGF-β1, TGF-β2 and TGF-β3) in 2000 cGy whole-lung irradiated C57BL/6J mice, a strain known to develop dose and volume-dependent organizing alveolitis/fibrosis. The results demonstrate increase in mRNA for IL-1 between days 1 and 14 after irradiation with return to baseline levels out to 120 days. TNF-α mRNA levels were not initially elevated but increased between 80 and 100 days and then decreased by 120 days. The mRNA levels for TGF-β1 demonstrated an initial increase within the first 14 days after total lung irradiation with a decrease to baseline levels out to 100 days. Then, in striking contrast to the other two cytokines, an increase in TGF-β2 mRNA occurred at around 120 days and correlated with the detection of organizing alveolitis/radiation fibrosis and mortality. These results are consistent with a two-phase mechanism in the molecular pathology of irradiation lung injury, in which IL-1 cytokine mRNA levels correlated with the acute pneumonitis phase and delayed elevation of TNF-α (80–100 days), TGF-β1 (100 days), and TGF-β2 (120 days) were associated with the fibrosis phase. Insight into the cell-specific and tissue-specific molecular mechanisms of ionizing irradiation induction of mRNA for pulmonary cytokines may provide new strategies for treatment of radiation pneumonitis in TBI patients.

KEY WORDS

Total body irradiation • Lung radiation injury • Gene therapy

INTRODUCTION

A major toxicity of total body irradiation (TBI) used in the preparation of patients for bone marrow transplantation is radiation pneumonitis [1–13]. Ionizing irradiation-induced lung damage has been shown to directly correlate with total dose [1], fraction size [12], and volume of lung irradiated [10]. In TBI patients, several strategies to decrease radiation pneumonitis have been attempted. These have included: decreasing the total dose with dose-rates from around 5 cGy/minute up to as high as 30 cGy/minute [10,15]; fractionating TBI with one or two fractions of around 125 cGy/day over 3 or 4 days [10,16–20]; and adding techniques of lung blocking either by using full thickness blocks on the second or third day of a 4-day treatment regimen or, alternatively, using a 50% transmission block for the entire 4 days of a fractionated program [10–11,19]. The techniques of lung and liver blocking have decreased pulmonary toxicity but result in a higher recurrence rate [8].

Pulmonary toxicity has been one motivation for new marrow transplant programs to clinically test alternative forms of patient preparation for marrow transplant that avoid irradiation, including the use of busulfan/cyclophosphamide or other chemotherapy regimens in the absence of TBI [21–24]. Limited success in using these alternatives in curing patients with acute myeloid leukemia in second or third remission [17, 24–25], T-cell leukemia [26], chronic myelogenous leukemia [18], multiple myeloma [27], or other disease categories in which TBI is utilized [27–28] suggest that this modality will have continued widespread appeal for use in new clinical protocols.

The mechanism of ionizing irradiation-induced lung injury is poorly understood, but has been shown to correlate with increased plasma levels of TGF-β [29] and other cytokines [29] associated with other forms of infectious or toxic tissue injury. The acute pathologic phase of irradiation lung injury includes detectable alveolar transudate and endothelial cell swelling [30]. A subacute phase includes inflammatory cell infiltrate and suggests some elements of common pathophysiology with pulmonary injury from hyperbaric oxygen or inhaled toxic chemicals [30–31].

Rodent models of radiation pneumonitis have been particularly helpful in elucidating the regulatory controls over both the acute and chronic phases of lung injury [30–34]. Several strains of mice have been demonstrated to develop a predictable pattern of organizing alveolitis/fibrosis, including the C57BL/6J mouse [31,33], while other strains appear resistant to this form of lung injury, including the C3H/HeJ mouse [29,34]. In back cross and F2 generation experiments, several genetic loci have been shown to be involved in the mechanism of susceptibility to radiation- induced organizing alveolitis [31,33–35]. Whether the critical irradiation tissue injury is sustained by alveolar type-two cells and tracheobronchial cells, bronchial endothelial cells, alveolar macrophages, or several cell types is not yet known [30,33,35]. Recent evidence suggests that levels of vascular endothelial growth factor increase after total lung irradiation in the C57BL/6J mouse [34]. These data point to initial injury in endothelial cells as perhaps critical to the cascade of events leading to radiation pneumonitis and subsequent radiation fibrosis.

Pharmacologic methods of radiation protection, including inhalation of acetylcysteine (Mucomyst) and other radical scavenger compounds, have been tested with limited success [36]. The use of inhaled antioxidant enzymes or radioprotective drugs has also been of limited success [37]. We have recently demonstrated the effectiveness of a method of radioprotective gene therapy using plasmid/liposome or adenovirus delivery of the manganese superoxide dismutase (MnSOD) transgene to the lungs of C57BL/6J mice [38–40]. These data show protection from acute radiation-induced cytokine increases, and a decrease in the appearance of organizing alveolitis/fibrosis and death at 150 days. The mechanism of pulmonary irradiation late effects and its correlation with acute effects is the subject of controversy. Corticosteroid treatment has been demonstrated to significantly decrease acute irradiation damage in the rat lung with minimal effect on late induction of fibrosis [41]. These data suggest a separate mechanism for the late effect of irradiation lung damage. In other models of radiation protection, intratracheal injection of basic fibroblast growth factor has been shown to decrease the initial, but not late, irradiation damage to the mouse lung [42].

Our prior studies did not address the events occurring in the lung between the acute and late injury. In the present study, we correlated the time course of appearance of organizing alveolitis in the mouse lung with levels of cytokine mRNA in total lung extracts at each of several intermediate time points. The experiments were carried out to determine whether the pathologic changes in the lung, which are predictably detected between 120 and 150 days in C57BL/6J mice after irradiation, correlated with the earlier appearance of a specific cytokine mRNA. If such a result were obtained and could point toward the appearance of a late irradiation injury, perhaps caused by apoptosis of a slowly proliferating cell population in the pulmonary parenchyma or vasculature, new interventional strategies might be designed. The results demonstrate a complex pattern of elevation of cytokine mRNA levels following total lung irradiation but with a clear indication that a late increase in the levels of two specific isoforms of TGF- β1 and TGF- β2 is reproducibly detected.

MATERIALS AND METHODS

Mice intratracheal liposome injections and irradiation

C57BL/6J female mice (30–33 gm) (Jackson Laboratories, Bar Harbor, ME) were intratracheally injected with 78 mL of either MnSOD plasmid liposome complex or LacZ plasmid/liposome complex. Mice were anesthetized with 1.25 mg of nembutal anesthesia/20 mg body weight (Abbott Laboratories, North Chicago, IL) as an intraperitoneal injection. The trachea was surgically exposed and a 1 cc syringe with a 28-gauge needle was used to inject into the trachea. The incision was closed with Autoclip 9.0 mm wound clips (Clay Adams, Parsippany, NJ). The pRK5 plasmid containing the human MnSOD cDNA as a transgene [43] and pIEP-LacZ plasmid [44] were grown in Luria-Bertani medium and isolated using Qiagen Giga columns (Qiagen, Chatsworth, CA). The plasmid DNA was resuspended in nuclease-free water at a concentration of 10 mg/ml. For each mouse to be injected, 500 mg of DNA (50 mL) and 28 mL of Lipofectin (Gibco/BRL, Grand Island, NY) were mixed and allowed to sit at room temperature for 30 minutes. The complexes were then placed on ice until the time of injection. Groups of 200 C57BL/6J mice received 2000 cGy to the pulmonary cavity one day after plasmid/liposome injection. All mice received single fraction irradiation to both lungs delivered by a 6 MeV linear accelerator Varian, dose rate 200 cGy/min at the mid-thoracic plane [38–39].The head and abdomen were shielded by ten $^1/_2$ value layer lead blocks.

Slot blots

For slot blots, 10 mg of total RNA was dissolved in 100 mL of diethyl pyrocarbonate (DEPC) (Sigma Chemical Company, St. Louis, MO) treated H_2O, followed by addition of 300 mL of a solution of 6.15*M* formaldehyde and 10× SSC. The RNA was incubated at 67°C for 15 minutes and loaded onto a Schleicher & Schuell Minifold II slot blot system (Schleicher & Schuell, Keene, NH), where the RNA is bound to BA85 nitrocellulose membrane (Schleicher & Schuell). The membrane was baked for 2 hours at 42°C, prehybridized in Denhardt's buffer (50% formamide, 5× Denhardt's

reagent, 100 mg/mL denatured salmon sperm DNA, 0.1% SDS, and 5× SSPE). The membrane was probed for 18 hours at 42°C with a p32-labeled PCR fragment of the MnSOD gene, then was washed twice in 1× SSPE/0.1% SDS for 5 minutes at room temperature followed by two washes in 0.1× SSPE/0.1% SDS at 42°C for 30 minutes. The blots were placed in an autoradiographic cassette with X-ray film. Densitometric analysis was carried out to determine the binding of the probe to the blot using a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). The membrane was stripped of radioactivity by placing the blot in 0.1× SSPE/0.1% SDS that had been heated to 100°C. After cooling to 65–70°C, the membrane was prehybridized as described above and reprobed for IL-1, TNF-α, TGF-β1, TGF-β2 and TGF-β3 with actin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech Laboratories, Palo Alto, CA) as standards. The cDNA probes for TGF-β1 (0.9 kb), TGF-β2 (1.0 kb) and TGF-β3 (0.75 kb) were a gift from Dr. Sonia B. Jakowlew of the Biomarkers and Prevention Research Division, National Cancer Institute and National Institutes of Health, Bethesda, MD. Densitometry was carried out as published and all results normalized separately using primer sets for actin and G3PDH.

Histopathology

For each treatment group, 5–10 mice were killed at each time point for both the slot blot analysis of cytokines and histopathological examination for irradiation-induced lung damage. The lungs were expanded by intratracheal injection of 0.8 mL of Optimum Cutting Temperature (OCT) compound, (Sakura Fine Tek, Torrance, CA) followed by excision. The five lobes of each mouse lung were separated, placed in each of five 15 × 15 mm base molds, and frozen in OCT [45]. Nine sections (each 10 microns thick) were prepared from each lobe with 100 microns between each section using a Shandon AS620E Cryotome (Shandon/Lipshaw, Pittsburgh, PA). The sections were hematoxylin and eosin (H&E)-stained as described [38]. The slides were dehydrated, mounted with Permamount (Fisher Scientific, Pittsburgh, PA), then visually scored for the percent of the lung exhibiting fibrosis and/or alveolitis [31]. For each mouse, at least seven sections were prepared from each of five lobes and scored separately. At least 36 total sections were analyzed by an Optimus Image Analysis System (Bothwell, WA) to quantitate the percent of lung involved with organizing alveolitis for each mouse. The method is to project the entire section on a computer monitor screen. Then with a hand-held pendant, each area of organizing alveolitis is traced and the area of each involved section is circled. The software algorithm calculates the percent of the total lung section surface area that is accounted for in the circled area, and this percent then is the percent alveolitis for that section. The automated scoring closely correlated with visual manual scoring from prior studies [38,40]. Irradiation-induced organizing alveolitis, the late histologic changes detected in C57BL/6J mice, were scored from H&E-stained sections [31]. The methods for collagen histochemical stains have been published previously and are shown in prior studies to correlate with percent organizing alveolitis detected by H&E stains in this strain [31].

Statistics

Standard survival analysis included Kaplan-Meier plots of survivor function and log rank tests of differences between treatment groups [46,47]. Prognostic factors, including radiation dose and radiation protection methods among genetically similar mice, were examined with Cox regression [48]. The association between alveolitis rates and survival was examined by adding mouse median alveolitis (fibrosis) rates as time-dependent covariates. Treatment group differences in alveolitis were tested by nonparametric methods; either the Wilcoxon test for two samples of the Kruskal-Wallis test, or the Jonckheere-Terpstra method for overall differences or trends [49–50] The group differences were applied to mouse median percent alveolitis.

Animal welfare

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Veterinary care was provided by the Central Animal Facility of the University of Pittsburgh in strict accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh guidelines.

RESULTS

Changes in cytokine mRNA levels over 120 days after whole lung irradiation

Our previous studies demonstrated that intratracheal injection of manganese superoxide dismutase (MnSOD) plasmid/liposome complex prior to whole lung irradiation decreased radiation-induced organizing alveolitis/fibrosis in C57BL/6J mice and improved survival [38,40]. The success of radioprotective gene therapy was correlated with detectable expression of transgene mRNA in alveolar type-two cells and tracheobronchial cells of treated mice, and with an elevated level of biochemically detectable MnSOD in explanted lung tissue 24 hours after gene therapy and immediately before irradiation. MnSOD plasmid/liposome gene therapy groups, compared to LacZ gene therapy irradiated mice or the irradiated control mice, demonstrated a decrease in the irradiation-induced levels of endogenous MnSOD in mouse lung, and also showed a decrease in irradiation-induced mRNA levels for TGF-β, IL-1, and TNF-α at early time points after irradiation [38,40].

A major question arising from our prior studies focused on the role of cytokine elevation following irradiation on the late effect of organizing alveolitis/fibrosis. Mice in our previous studies were followed until the time of death, and histopathology with quantitation of the percent of lung containing organizing alveolitis was evaluated at death or when mice were moribund. No information was available from these first experiments on whether chronic elevation of cytokine levels or delayed peak elevation of specific inflammatory or fibrosis-associated cytokine levels was correlated with the severity of organizing alveolitis, nor could we determine whether the single administration of MnSOD plasmid/liposomes 24 hours prior to irradiation had altered the late expression of cytokine mRNA levels in the lung.

In the present studies, 600 mice were divided into three groups receiving daily administration of MnSOD plasmid/liposome gene therapy, LacZ plasmid/liposome control transgene

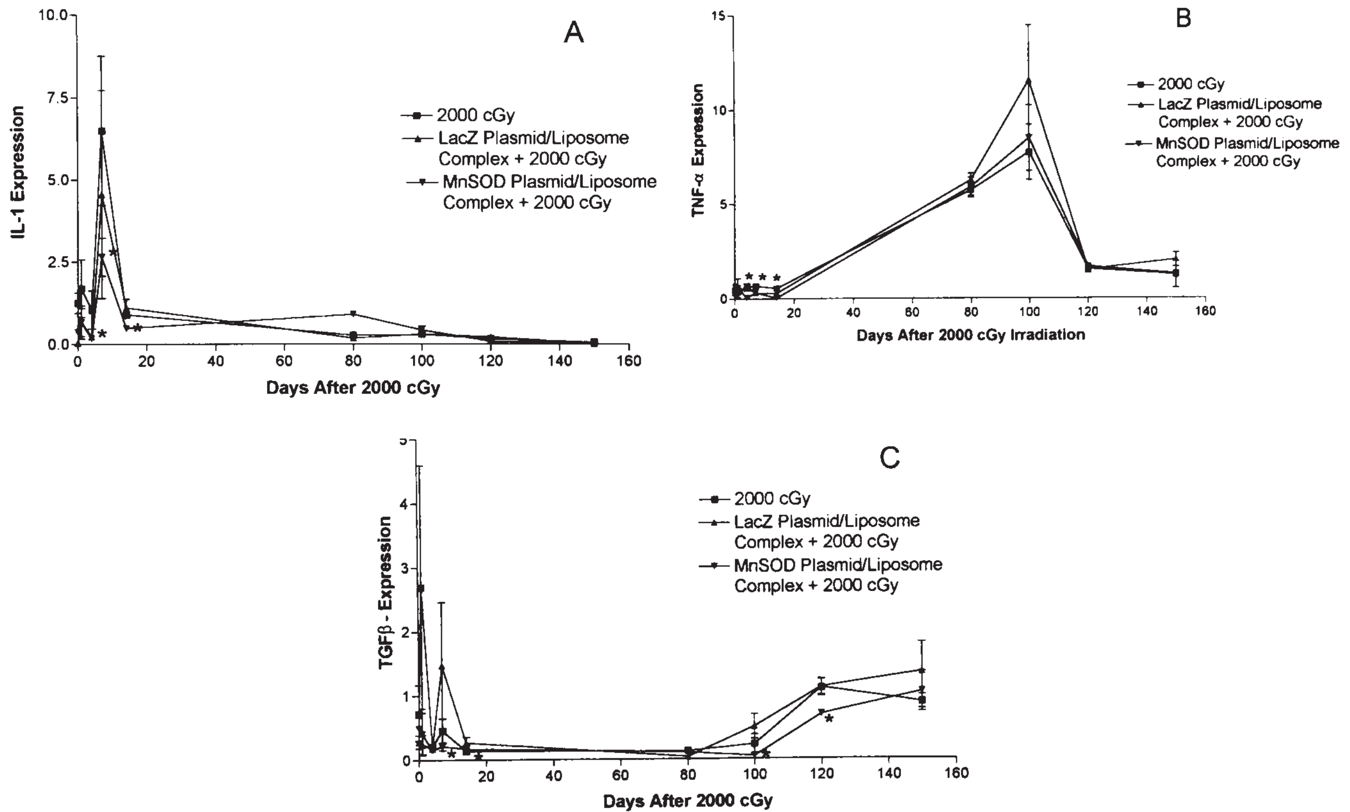

Figure 1. Increased cytokine expression in the lung following 2000 cGy irradiation

Control C57BL/6J mice, as well as mice injected intratracheally with either LacZ plasmid/liposome complex or MnSOD plasmid/liposome complex 24 hours previously, were irradiated to a dose of 2000 cGy to the pulmonary cavity. At 0, 1, 4, 7, 14, 80, 100, or 120 days following irradiation or at the time of death, the mice were sacrificed, and the lungs were removed and snap frozen in liquid nitrogen. RNA was isolated and slot blot analysis was performed. The blots were probed with a p32-labeled PCR fragment for IL-1 (A), TNF-α (B), or TGF-β (C), and then stripped and reprobed with a p32-labeled PCR fragment of actin. The RNA was quantitated spectrophotometrically and data reported as cytokine:actin ratios to normalize for RNA loading. There was an increase in IL-1 expression on days 1 and 7 following irradiation (A). For TNF-α (B), there was an increase at 80 and 100 days after irradiation, but decreases at 120 days and at the time of death. There was an initial increase in TGF-β (C) following irradiation, which returned to background levels by day 14. At 100 days, there was an increase in TGF-β expression which continued to increase to 120 days and at the time of death. Cytokine levels for mice injected with the MnSOD plasmid/liposome complex were compared to control mice using a Student t-test (significant differences with a $p < 0.05$ are indicated by an asterisk [*]).

administration, or no plasmid/liposome injection, 24 hours prior to 2000 cGy whole lung irradiation. The 2000 cGy dose was chosen based on our previous studies and was higher than the 1800 cGy or 1900 cGy dose [38]. This higher dose was chosen to attempt to amplify cytokine mRNA changes and to provide the optimum ability to correlate potential early irradiation-induced histopathologic changes with cytokine mRNA levels. For this large study, a subgroup of mice was followed until death and seven other subgroups from each main group were sacrificed at days 1, 4, 7, 14, 80, 100, or 120 after whole lung irradiation. These groups of sacrificed animals were evaluated for levels of mRNA for IL-1, TGF-β (and isoforms TGF- β1, TGF- β2 and TGF- β3), and TNF-α in whole lung tissue immediately after explant and fragments of lung from each of the five lobes were analyzed rigorously for histopathologic evidence of organizing alveolitis according to our published methods [38,40].

C57BL/6J mice were intratracheally injected with either LacZ or MnSOD plasmid/liposome complex and irradiated 24 hours later, along with control mice, to 2000 cGy to the pulmonary cavity. The mice were sacrificed at various time points following irradiation or at time prior to death. RNA was extracted from the lungs, which were excised and frozen in liquid nitrogen. Slot blot analysis was used to quantitate the expression of mRNA for IL-1 by probing the blots with a p32-PCR fragment of IL-1, stripping the blot and probing for actin. Expression was measured by densitometric measurements of the x-ray film from the blots with the results normalized by comparing IL-1 expression with actin and, separately, with G3PDH expression.

Lung specimens were analyzed for levels of mRNA for IL-1, TNF-α, and each of three TGF-β isoforms from subgroups of at least five mice at each time point. At least five samples of lung were evaluated in each cytokine assay by slot blot and the results normalized for each fragment of lung to actin and G3PDH content in that fragment. The results were presented as the mean and standard deviation for each mouse and then mean and standard error for each group of five mice. As shown in Fig. 1, irradiated control mice as well as those receiving LacZ plasmid/liposome

Table 1. *Expression of IL-1 mRNA in the lung following 2000 cGy irradiation normalized to G3PDH*

	Days After 2000 cGy Irradiation				
Group	**0**	**80**	**100**	**120**	**Death**
Control	**0.341 ± 0.037**	**0.329 ± 0.026**	**0.848 ± 0.227**	**1.201 ± 0.341**	**0.361 ± 0.050**
LacZ	**0.186 ± 0.087**	**0.777 ± 0.245**	**0.781 ± 0.233**	**0.763 ± 0.143**	**0.487 ± 0.255**
MnSOD	**0.370 ± 0.087**	**0.071 ± 0.006***	**0.620 ± 0.155**	**0.221 ± 0.089***	**0.210 ± 0.020**

C57BL/6J mice were injected with either MnSOD or LacZ plasmid/liposome complex. Control mice, as well as mice injected with MnSOD or LacZ plasmid/liposome complex were irradiated to the pulmonary cavity to 2000 cGy, 24 hours after injection with MnSOD or LacZ plasmid/liposome complex. The mice were sacrificed on days 0, 80, 100, and 120 after irradiation, and the lungs removed and frozen in liquid nitrogen. RNA was extracted and slot blot analysis performed to quantitate mRNA expression of IL-1 by probing with a p32-labeled PCR fragment for IL-1. The blots were stripped and reprobed with a p32-labeled probe G3PDH. Expression was measured by densitometric measurements of the x-ray film from the blots with IL-1 expression normalized by comparison to G3PDH expression. The above results are IL-1:G3PDH ratios. A Student t-test was used to compared IL-1 expression for MnSOD plasmid/liposome-injected mice to the control irradiated mice with significant differences ($p < 0.05$) indicated by an asterisk (*). IL-1 expression was significantly decreased in mice injected with MnSOD plasmid/liposome complex 80 and 120 days after irradiation. These differences were not detected when data was normalized to actin (Figure 1A).

demonstrated a significant increase in mRNA levels for IL-1, detectable as early as 1 day after irradiation and significantly elevated at 7 days after irradiation. IL-1 mRNA levels decreased after day 7 and did not rise again significantly over the 120-day observation period and were not elevated at death. While there was no statistically significant difference between IL-1 mRNA levels in the LacZ plasmid/liposome group compared to irradiated controls, MnSOD plasmid/liposome administration prior to irradiation significantly decreased the irradiation-induced elevation of IL-1 mRNA at days 4, 7, and 14 after irradiation (Fig. 1A). When the data were normalized to G3PDH (Table 1), the irradiation-induced increase was again detectable, and a significant decrease in irradiation-induced upregulation of IL-1 in the MnSOD plasmid/liposome group was detected at days 80 and 120 (Table 1). These results confirm and extend those of our prior studies [38] and are consistent with the observation that IL-1 is an acute phase inflammatory cytokine responding to pulmonary damage resulting from infection, chemical toxicity, or exposure to DNA damaging agents.

The results with mRNA levels for TNF-α at each of the time points evaluated are shown in Fig. 1B. The expression of TNF-α was determined by spectrophotometrical analysis of X-ray films of the blots with the expression of the RNA normalized by comparing to actin expression with the results shown above as TNF-α:actin ratios. A Student *t*-test was used to compare TNF-α expression in MnSOD plasmid/liposome complex-injected mice with control mice and mice injected with LacZ, and significant differences were detected at days 4, 7, and 14 ($p < 0.05$). Irradiated control mice demonstrated an increase in TNF-α mRNA levels detectable at day 4 and significantly increasing at days 80 and 100. Mice injected with MnSOD plasmid/liposome complex prior to irradiation showed significantly decreased TNF-α mRNA levels at days 4, 7, and 14 after irradiation. There was then a significant decrease in TNF-α mRNA levels at 120 days and at the time of death, compared to those levels detected at 80 and 100 days. These results were similar for the mice receiving LacZ plasmid/liposome administration prior to irradiation. In the mice receiving MnSOD plasmid/liposome gene therapy prior to irradiation, there was a significant decrease in irradiation-induced mRNA levels for TNF-α detectable at days 4, 7, and 14 after irradiation.

When the data were normalized to G3PDH, we detected persistently elevated levels of TNF-α mRNA after irradiation out to day 120, and a significant decrease in TNF-α levels of mRNA at 120 days in the MnSOD plasmid/liposome group (Table 2). These results further confirm and extend our prior

Table 2. *Expression of TNF-a in the lung following 2000 cGy irradiation normalized to G3PDH*

	Days After 2000 cGy Irradiation				
Group	**0**	**80**	**100**	**120**	**Death**
Control	**5.912 ± 0.5191**	**7.252 ± 1.258**	**15.78 ± 0.043**	**11.42 ± 1.601**	**7.141 ± 1.139**
LacZ	**4.286 ± 1.030**	**19.15 ± 2.784**	**18.86 ± 2.249**	**6.750 ± 0.798**	**11.24 ± 3.322**
MnSOD	**5.647 ± 1.437**	**12.72 ± 1.683**	**12.84 ± 1.610**	**5.176 ± 0.988***	**9.478 ± 2.149**

Control C57BL/6J mice, as well as mice which had been injected intratracheally 24 hrs previously, were irradiated to 2000 cGy to the pulmonary cavity. Following irradiation, the mice were sacrificed at days 0, 80, 100, 120 and time of death, and the lungs excised and frozen in liquid nitrogen. RNA was extracted and slot blot analysis was performed to determine the expression of TNF-α following irradiation. The blots were probed with a p32-labeled PCR fragment of TNF-α, and stripped and probed with a p32 probe for G3PDH. The expression of TNF-α was determined by spectrophotometric analysis of x-ray film of the blots with the expression normalized by comparing G3PDH expression. Results are shown as TNF-α:G3PDH ratios. A Student t-test was used to compare TNF-α expression in MnSOD plasmid/liposome complex-injected mice with control mice and mice injected with LacZ plasmid/liposome complex with significant differences ($p<0.05$) indicated by an asterisk (*). Mice injected with MnSOD plasmid/liposome complexes showed significantly lower levels of TNF-α mRNA at 120 days after irradiation, compared to the control irradiated mice. The effect of MnSOD gene therapy on decrease in TNF-α at 120 days was not detected when the data was normalized to actin (Figure 1B).

Table 3. *Expression of TGF-β in the lung following 2000 cGy irradiation normalized to G3PDH*

Group	Days After 2000 cGy Irradiation				
	0	80	100	120	Death
Control	**0.105 ± 0.011**	**0.434 ± 0.254**	**0.474 ± 0.175**	**8.305 ± 1.464**	**5.813 ± 1.355**
LacZ	**0.202 ± 0.077**	**0.119 ± 0.049**	**0.827 ± 0.242**	**4.804 ± 0.844**	**7.641 ± 3.044**
MnSOD	**0.135 ± 0.017**	**0.205 ± 0.083**	**0.063 ± 0.025***	**2.305 ± 0.266***	**3.913 ± 1.056**

Control C57BL/6J mice, as well as mice intratracheally injected 24 hours previously with either MnSOD plasmid/liposome complex or LacZ plasmid/liposome, were irradiated to 2000 cGy. The mice were sacrificed at 0, 80, 100, or 120 days after irradiation or at the time of death, and the lungs excised and snap frozen in liquid nitrogen. RNA was extracted and expression of mRNA for TGF-β was measured using slot blot analysis. The blots were probed with a p32-PCR fragment for TGF-β, and stripped and probed with a p32-PCR fragment for G3PDH. The expression of TGF-β was normalized by comparing G3PDH with the results shown as TGF-β:G3PDH ratios. A Student t-test was used to compare the control mice and mice injected with LacZ plasmid/liposome complex with the MnSOD plasmid/liposome-injected mice (significant differences with a $p < 0.05$ indicated by an asterisk [*]). Mice injected with MnSOD plasmid/liposome complex showed significantly decreased TGF-β mRNA at days 100 and 120 after irradiation.

data on the effects of MnSOD plasmid/liposome gene therapy on cytokine mRNA induction in the lung at these earlier time points [38]. Mice in the MnSOD plasmid/liposome group demonstrated a late elevation in TNF-α mRNA at days 80 and 100, and a decrease at day 120 and at death. There was a significant effect of MnSOD plasmid/liposome gene therapy with respect to lowering postirradiation TNF-α levels at earlier time points, and the late sustained elevation in TNF-α and drop in levels prior to the time of death was also detectably decreased at day 120 by this schedule of MnSOD plasmid/liposome delivery.

The results of detection of mRNA levels for total TGF-β and each of three isoforms of TGF-β in the lungs of mice in each group are shown in Fig. 1C and in Tables 3–6. The expression of each TGF-β isoform was normalized by comparing it to G3PDH (Tables 4–6). The results with total TGF-β are presented as TGF-β:actin ratios (Fig. 1C) and TGF-β:G3PDH ratios (Table 3). A Student *t*-test was used to compare the control mice and mice injected with LacZ plasmid/liposome complex with mice injected with MnSOD plasmid/liposome. Control irradiated mice demonstrated a significant increase compared to preirradiation levels in TGF-β at 1 day after irradiation followed by a decrease to levels similar to that prior to irradiation. A late increase in total TGF-β levels at 120 days and at the time of death was detected (Fig. 1C, Table 3). The late peak increase in elevation of TGF-β was clearly different in its pattern from that of the decrease prior to death, compared to TNF-α levels (Fig. 1B). Mice injected with MnSOD plasmid/liposome complex showed significantly decreased ($p < 0.05$) levels of total TGF-β mRNA at days 7, 14, 100, and 120 after irradiation (late significant differences with a $p < 0.05$ are indicated by an asterisk [*]) (Fig. 1C; Table 3). The results of measurement of mRNA levels for each TGF-ß isoform (Tables 4–6) were similar in the groups receiving irradiation alone or LacZ plasmid/liposome administration prior to irradiation with respect to the late elevation of TGF-ß1 at 100 days, and TGF-ß2 at day 120 and at the time of death. In contrast, mice receiving MnSOD plasmid/liposome gene therapy demonstrated a significant decrease in total TGF-β levels (Fig. 1C, Table 3) and TGF-β1 levels (Table 4), compared to control irradiated or LacZ plasmid/liposome-treated animals at days 7 and 14, and at the late elevation points of 100 days for TGF-β1, and 120 days for TGF-β2 (Tables 4 and 5, respectively). Results with measurement of TGF-β2 and TGF-β3 normalized to G3PDH are shown in Tables 5 and 6, respectively. There was no detectable change in TGF-β3 levels at any time point.

While there was a significant decrease in TGF-β mRNA levels at 120 days in the MnSOD plasmid/liposome-treated group, the levels of mRNA in lung tissue at the time of death were not significantly lower than those in the other groups. While mRNA levels within each group at each time point had large standard error bars for some data points (example: day 0 for TGF-β irradiated control group), the patterns of difference in mRNA levels normalized to actin or G3PDH clearly

Table 4. *Expression of TGF-β1 mRNA in the lung following 2000 cGy irradiation normalized to G3PDH*

Group	Days After 2000 cGy Irradiation				
	0	80	100	120	Death
Control	**0.065 ± 0.027**	**0.001 ± 0.001**	**0.163 ± 0.053**	**0.015 ± 0.009**	**0.021 ± 0.011**
LacZ	**0.006 ± 0.004**	**0.025 ± 0.012**	**0.094 ± 0.045**	**0.016 ± 0.006**	**0.047 ± 0.019**
MnSOD	**0.020 ± 0.001**	**0.010 ± 0.004**	**0.059 ± 0.008***	**0.020 ± 0.004**	**0.012 ± 0.004**

C57BL/6J mice were injected intratracheally with either MnSOD or LacZ plasmid/liposome complex. Twenty-four hours later, control and injected mice were irradiated to 2000 cGy to the pulmonary cavity. The mice were sacrificed on days 0, 80, 100, and 120 following irradiation, or at the time of death, and the lungs excised and snap frozen in liquid nitrogen. The RNA was extracted and analyzed for expression of TGF-β1 mRNA by slot blot analysis. The blots were probed with a p32-labeled fragment of the rat TGF-β1 gene, which recognizes the murine TGF-β1 mRNA. The blots were stripped and reprobed with a p32-labeled PCR fragment for G3PDH. The expression of TGF-β1 was normalized to G3PDH expression with the results shown as TGF-β1:G3PDH ratios. A Student t-test was used to compare the expression of MnSOD plasmid/liposome complex-injected mice to that of irradiated control mice (significant differences with a $p < 0.05$ are indicated by an asterisk [*]). Expression of TGF-β1 increased 100 days after irradiation and then decreased by 120 days. MnSOD plasmid/liposome-injected mice had a significantly lower expression of TGF-β1 at 100 days, compared to the control irradiated mice.

Table 5. *Expression of TGF-ß2 mRNA in the lung following 2000 cGy irradiation normalized to G3PDH*

Group	Days After 2000 cGy Irradiation				
	0	80	100	120	Death
Control	0.081 ± 0.011	0.064 ± 0.026	0.067 ± 0.034	0.273 ± 0.026	0.277 ± 0.533
LacZ	0.050 ± 0.013	0.109 ± 0.032	0.039 ± 0.011	0.182 ± 0.026	0.243 ± 0.042
MnSOD	0.082 ± 0.013	0.067 ± 0.006	0.023 ± 0.003	0.108 ± 0.027*	0.502 ± 0.159

Control C57BL/6J mice, as well as mice that had been intratracheally injected with either MnSOD or LacZ plasmid/liposome complex 24 hrs previously, were irradiated to 2000 cGy to the pulmonary cavity. At 0, 80, 100, 120 days after irradiation, as well as at the time of death, the mice were sacrificed with the lungs excised and frozen in liquid nitrogen. RNA was extracted and slot blot analysis was performed to determine the expression of TGF-ß2 mRNA. The blots were probed with a p32-labeled fragment of the murine TGF-ß2 gene, and stripped and probed with a p32-labeled G3PDH PCR fragment. The expression of TGF-ß2 mRNA was determined by spectrophotometrical analysis of x-ray films with the expression normalized by comparison to the expression of G3PDH, with results presented as TGF-ß2:G3PDH ratios. A Student t-test was used to compare the expression of TGF-ß2 expression of MnSOD plasmid/liposome complex-injected mice to that of the control mice [significant differences of p<0.05 are indicated by an asterisk (*)]. TGF-ß2 expression increased at 120 days after irradiation and remained elevated at the time of death. Mice injected with MnSOD plasmid/liposome complex had a significantly lower expression of TGF-ß2 at 120 days than the control or LacZ plasmid/liposome complex-injected mice.

demonstrated a difference in the kinetics of appearance of IL-1, TNF-α and TGF-β isoform mRNA levels, which were uniform between groups and uniform in peak elevation at specific time points. These data establish that pulmonary IL-1 mRNA levels increased initially after total lung irradiation and fell to lower levels until the time of death. They also demonstrate that a progressive increase in TNF-α mRNA levels after irradiation was associated with a decrease around the time that a second peak elevation of TGF-β1 and TGF-β2 isoform levels was detected in all groups.

Histopathologic changes associated with organizing alveolitis/fibrosis are detected after 120 days following total lung irradiation

Rigorous histopathologic evaluation of all five lobes from each lung of each individual mouse within each subgroup sacrificed at days 80, 100, and 120 were compared to the results of histopathologic evaluation at the time of death. Our previous studies of total lung irradiation in the C57BL/6J mouse have demonstrated that there is no reliable physiologic measurement prior to morbidity and death in this strain, suggesting the histopathologic changes occurred rapidly before the time of death [38]. To determine whether detectable changes in histopathology were present at earlier time points, each subgroup was carefully compared with each other group and with representatives from its own group at different time points.

As shown in Fig. 2, there was no detectable increase in the percent of lung containing histopathologically detectable organizing alveolitis at 80 days or 100 days after total lung irradiation compared to control levels in the present study (data not shown) or studies published by us previously [38]. In contrast, at 120 days, there was an increase in percent alveolitis in the MnSOD plasmid/liposome gene therapy

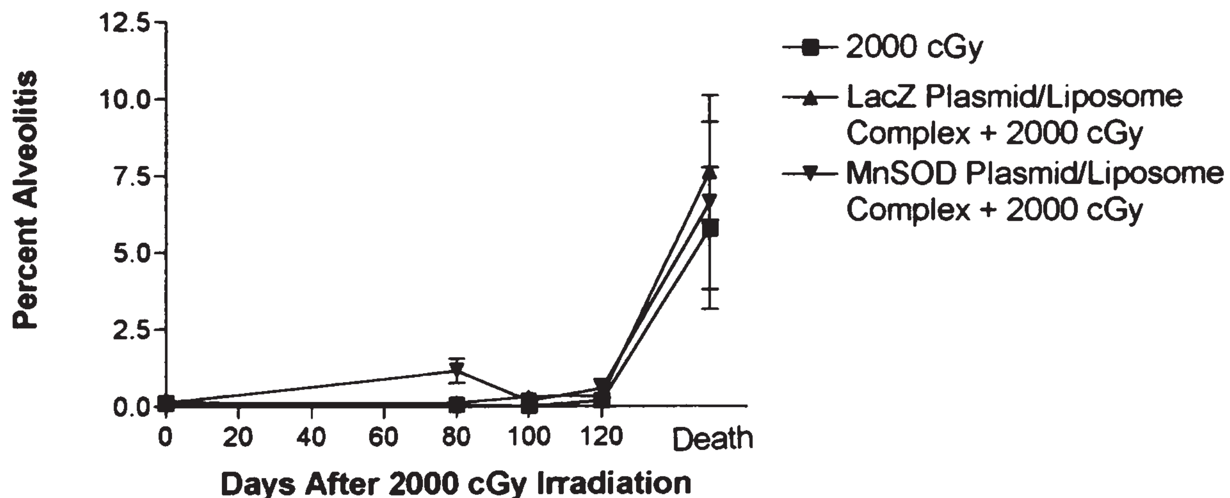

Figure 2. Percent alveolitis in the lungs following irradiation

Control C57BL/6J mice, as well as mice injected intratracheally with either LacZ plasmid/liposome complex or MnSOD plasmid/liposome complex 24 hours previously, were irradiated to 2000 cGy to the pulmonary cavity. The mice were sacrificed at 80, 100, or 120 days after irradiation or at the time of death, and the lungs excised, frozen in OCT, sectioned and H&E stained. The percent alveolitis was determined using the Optimus Image Analysis System. No significant increase in alveolitis was detected before the time of death.

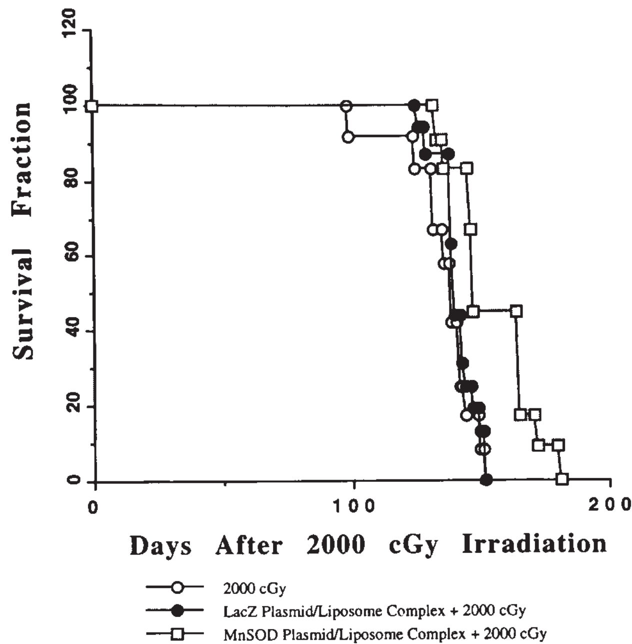

Figure 3. Increased survival of MnSOD plasmid/liposome complex-injected mice irradiated to 2000 cGy

C57BL/6J mice were injected intratracheally with MnSOD or LacZ plasmid/liposome complexes. The injected mice, as well as control mice were irradiated to 2000 cGy to the pulmonary cavity 24 hours later and followed for survival. The mice injected with MnSOD plasmid/liposome complex had a significantly increased survival compared to the control and LacZ plasmid/liposome complex-injected mice (p = 0.003 and 0.006, respectively).

group and a significant increase in all three groups at death (Fig. 2, Table 7). As shown in Fig. 3, there was a significant increase in survival in the MnSOD plasmid/liposome gene therapy group, compared to the 2000 cGy irradiated control or LacZ plasmid/liposome complex-treated groups. These results confirm and extend those of our prior studies using lower single-fraction doses of irradiation [38]. The earlier death of mice in the control irradiated group between days 100 and 150 may be considered when analyzing the data in Table 1 showing percent organizing alveolitis. The percent alveolitis of 5.800 ± 0% in the control group reflected unprotected mice who died significantly earlier than those in the MnSOD plasmid/liposome group or the LacZ plasmid/liposome group, even though the group composite number for percent alveolitis appeared to be higher in the plasmid/liposome-treated groups.

These results establish that the appearance of organizing alveolitis correlated with the second peak increase in mRNA levels for specific isoforms of TGF-β1 and TGF-β2. The data further indicate that there was no detectable appearance of histopathologic changes associated with organizing alveolitis or fibrosis at 100 days, and in two of the groups at 120 days (Table 7).

DISCUSSION

Pulmonary toxicity from TBI remains a major complication in the preparative regimens for bone marrow transplantation [1–7,10–12,14–15]. The pathophysiology of radiation pneumonitis and late radiation fibrosis are largely unknown, but the sequelae of each presentation have led to speculation as to the etiology of the causes of toxicity in TBI patients. Increased levels of cytokine mRNA and plasma protein levels for IL-1, TNF-α< and TGF-β have been associated with total lung irradiation and have been suggested as contributing to the radiation pneumonitis seen in TBI patients [29,31,34,42,51]. Elevated cytokine levels producing inflammatory cell reactions in the lung have been suggested as etiologic agents in pulmonary toxicity [10]. Opportunistic infection resulting from the immunosuppression of TBI and/or elevated cytokine levels has also been suggested [10,28,38,40]. Finally, pulmonary vascular damage and the TBI effect of upregulation of vascular adhesion molecules has been suggested as a potential etiologic agents in radiation pneumonitis [34,52].

Lung shielding using transmission blocks and fractionated radiotherapy have significantly decreased the acute toxicity of whole body irradiation with respect to radiation pneumonitis [8,18,22,27–28]. However, the worrisome increase in relapse of myelodysplastic disease in patients with lung shielding [8] suggests that protection of bone marrow in ribs andvertebral bodies by the lead blocks may be an undesirable outcome of this method of lung protection. Pharmacologic approaches to lung protection have been attempted utilizing agents that have worked *in vitro* or

Table 6. *Expression of TGF-β3 in the lung following 2000 cGy irradiation normalized to G3PDH*

Group	Days After 2000 cGy Irradiation 0	80	100	120	Death
Control	**0.042±0.004**	**0.044±0.006**	**0.017±0.007**	**0.031±0.011**	**0.033±0.003**
LacZ	**0.054±0.030**	**0.053±0.013**	**0.045±0.013**	**0.058±0.012**	**0.058±0.020**
MnSOD	**0.024±0.005**	**0.048±0.009**	**0.011±0.005**	**0.014±0.003**	**0.028±0.016**

Control C57BL/6J mice, as well as mice which had been injected 24 hrs previously with either MnSOD or LacZ plasmid/liposome complex, were irradiated to 2000 cGy to the pulmonary cavity. On days 0, 80, 100, and 120 after irradiation, as well as at the time of death, the mice were sacrificed and the lungs excised and frozen in liquid nitrogen. The RNA was extracted and analyzed for the expression of the TGF-β3 isoform using slot blot analysis. The blots were probed with a p32-labeled fragment of the murine TGF-β3 gene, and stripped and reprobed with a p32-labeled PCR fragment of the G3PDH gene. Expression of TGF-β3 was determined by spectrophotometrical analysis of X-ray films of the blots with the expression normalized by comparison of the expression of G3PDH, with results presented as TGF-β3:G3PDH ratios. A Student t-test was used to compare the expression of TGF-β3 in the MnSOD plasmid/liposome complex-injected mice to that of the control irradiated mice. There was no significant increase in expression of TGF-β3 at any time following irradiation. There also was no change in expression of TGF-β3 in any of the different groups of mice following 2000 cGy irradiation.

Table 7. *Percent alveolitis following 2000 cGy to the mouse lung*

Group	Days After 2000 cGy Irradiation 80	100	120	Death
Control	**0.077 ± 0.1**	**0.041 ± 0.04**	**0.206 ± 0.2**	**5.800 ± 2.0***
LacZ	**0.320 ± 0.2**	**0.359 ± 0.2**	**0.194 ± 0.1**	**7.681 ± 1.6***
MnSOD	**0.133 ± 0.1**	**0.20 ± 0.1**	**0.593 ± 0.3**	**6.654 ± 3.5***

Control C57BL/6J mice, as well as mice which had been injected 24 hours previously with either MnSOD or LacZ plasmid/liposome complex, were irradiated to 2000 cGy to the pulmonary cavity. On days 80, 100, and 120 after irradiation or at the time of death, the mice were sacrificed; and the were lungs excised, frozen in OCT, sectioned and H&E-stained with the percent alveolitis calculated using an Optimus Image Analysis System [31]. No alveolitis was detected before the time of death. There was no significant difference in the percent of alveolitis among the three groups of mice; however, all showed a significant increase in alveolitis at death, compared to values at earlier time points within each group ($p < 0.001$).

in the oral cavity with limited success, perhaps due to unavailability of the agents to the critical pulmonary cells or to their nuclei [53–54].

The mouse model of total lung irradiation is not an ideal one for studying acute radiation pneumonitis in humans because the doses which produce an acute pulmonary effect are beyond those associated with lethality from esophagitis at 30 days [30]. In other studies, we have demonstrated significant protection of the mouse esophagus from irradiation-induced lethal esophagitis by MnSOD plasmid/liposome gene therapy, and this method of promoting survival of mice to doses above 3000 cGy whole lung irradiation may provide a mechanism with which to study cytokine levels in the lung following whole thorax irradiation under conditions where animals will not die at 30 days due to esophagitis. However, in the current analogous system to whole lung irradiation of TBI, doses between 1800 cGy and 2000 cGy in the C57BL/6J model appear to be ideally suited for studying cytokine levels associated with the late lung irradiation.

While mice in the present 2000 cGy irradiated control group did not die of radiation pneumonitis, these mice did present an effective model for study of the cytokine mRNA levels associated with late radiation fibrosis/organizing alveolitis. The present results established that there was a slow increase in detectable mRNA levels for TNF-α out to 100 days following total lung irradiation, followed by a drop to control pre-radiation levels. The different survival rates of control irradiated non-MnSOD plasmid/liposome-treated mice in the present versus past studies reflect the use of different animal care facilities [38] and C57BL/6J mice from different breeding stocks. In contrast, a late peak elevation in mRNA levels for specific isoforms of TGF-β was detected between 100 and 120 days, and was significantly elevated at the time of death. These results strongly suggest that TGF-β mRNA levels and protein production in the lung are associated with the late and death-related lesion of organizing alveolitis/fibrosis.

We did not measure actual cytokine protein levels in the lung, and do not know which cells of the pulmonary parenchyma are involved in the increased production of protein which was associated with elevated mRNA levels and serum protein levels [38]. Studies in progress with bronchoalveolar lavage to isolate pulmonary macrophages should allow us to determine if these and other inflammatory cells are involved in the irradiation-induced upregulation of cytokine message levels and protection by MnSOD plasmid/liposome therapy.

It is not known why the late elevation of specific TGF-β isoforms is associated with the histopathologic lesion of fibrosis, or why the increased level of TGF-β1 initially at days 4–14 does not also rapidly induce fibrosis early after irradiation. It is known that TGF-β has many different and pleotrophic functions in the developing embryo and in the adult [51]. Since our assay measured total levels of TGF-β and only subgroups of TGF-β1, TGF-β2, TGF-β3 [56], it is possible that the late peak elevation of TGF-β2 may represent one of several molecular species different from that detected in the earlier time points.

The presence of elevated levels of one cytokine in the absence of elevated levels of other cytokines may produce a very different response compared to elevation uniformly of multiple cytokine levels. It is possible that in the 120-day post irradiation lung, the elevated levels of specific TGF-β isoforms in the absence of associated elevated IL-1, TNF-α (or other cytokine levels which we did not study) may produce the fibrosis-inducing effect. It is also possible that the levels of some TGF-β isoform mRNAs detected after 100 days in the irradiated lung may reflect a damage response from slowly proliferating cells within the lung which required 120 days for cellular turnover and delayed apoptosis. Histopathologic and histomorphometric studies using *in situ* nested RT-PCR assays will be required to detect which cells in the 120-day post irradiation lung are responding with the increased levels of TGF-β2 mRNA.

The complication of radiation damage from TBI remains a major obstacle to successful attempts to improve the preparatory regimen for allogeneic bone marrow transplantation. It is an obstacle that must be overcome and such efforts must take into account the need to deliver a full dose to rib, clavicle, scapula, vertebral body, and sternal bone marrow, where residual leukemia lymphoma cells may reside [8]. Furthermore, successful strategies for protection of the lung should not protect lymph nodes in the mediastinum or in the pulmonary parenchyma, which also may harbor malignant cells. A strategy of radioprotective gene therapy using direct intrapulmonary delivery of MnSOD plasmid/liposome radioprotective genes must include a more complete understanding of the molecular mechanism of protection. The present studies add insight into the nature of the duration of protection, but more importantly into the profile of cytokine mRNA expression during the induction of radiation damage.

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