Transforming Growth Factor Alpha is a Critical Mediator of Radiation Lung Injury

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Radiation fibrosis of the lung is a late toxicity of thoracic irradiation. Epidermal growth factor (EGF) signaling has previously been implicated in radiation lung injury. We hypothesized that TGF- α , an EGF receptor ligand, plays a key role in radiation-induced fibrosis in lung. Mice deficient in transforming growth factor (TGF- $\alpha^{-/-}$) and control C57Bl/6J (C57-WT) mice were exposed to thoracic irradiation in 5 daily fractions of 6 Gy. Cohorts of mice were followed for survival ($n \geq 5$ per group) and tissue collection ($n = 3$ per strain and time point). Collagen accumulation in irradiated lungs was assessed by Masson's trichrome staining and analysis of hydroxyproline content. Cytokine levels in lung tissue were assessed with ELISA. The effects of TGF- α on pneumocyte and fibroblast proliferation and collagen production were analyzed *in vitro*. Lysyl oxidase (LOX) expression and activity were measured *in vitro* and *in vivo*. Irradiated C57-WT mice had a median survival of 24.4 weeks compared to 48.2 weeks for irradiated TGF- $\alpha^{-/-}$ mice ($P = 0.001$). At 20 weeks after irradiation, hydroxyproline content was markedly increased in C57-WT mice exposed to radiation compared to TGF- $\alpha^{-/-}$ mice exposed to radiation or unirradiated C57-WT mice (63.0, 30.5 and 37.6 $\mu\text{g/lung}$, respectively, $P = 0.01$). C57-WT mice exposed to radiation had dense foci of subpleural fibrosis at 20 weeks after exposure, whereas the lungs of irradiated TGF- $\alpha^{-/-}$ mice were largely devoid of fibrotic foci. Lung tissue concentrations of IL-1 β , IL-4, TNF- α , TGF- β and EGF at multiple time points after irradiation were similar in C57-WT and TGF- $\alpha^{-/-}$ mice. TGF- α in lung tissue of C57-WT mice rose rapidly after irradiation and remained elevated through 20 weeks. TGF- $\alpha^{-/-}$ mice had lower basal LOX expression than C57-WT mice. Both LOX expression and LOX activity were increased after irradiation in all mice but to a lesser degree in TGF- $\alpha^{-/-}$ mice. Treatment of NIH-3T3 fibroblasts with TGF- α resulted in increases in proliferation, collagen production and LOX

activity. These studies identify TGF- α as a critical mediator of radiation-induced lung injury and a novel therapeutic target in this setting. Further, these data implicate TGF- α as a mediator of collagen maturation through a TGF- β independent activation of lysyl oxidase. © 2014 by Radiation

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INTRODUCTION

Normal tissue toxicity is a dose-limiting factor in the therapeutic application of ionizing radiation. Thoracic irradiation can result in both pneumonitis and fibrosis. Inflammatory cell infiltration, proinflammatory cytokine production, myofibroblast proliferation and extensive collagen production are characteristic of progressive radiation-induced pulmonary fibrosis (1).

Epidermal growth factor receptor (EGFR) signaling plays an important role in epithelial proliferation and homeostasis (2–5), processes implicated in the initiation and perpetuation of fibrotic lung pathologies. Increased EGFR expression and activation have been described in a number of fibrotic lung pathologies, including radiation fibrosis (6, 7). Conversely, little is known about the expression of EGFR ligands in the setting of radiation lung injury.

The aim of the current study was to determine the importance of transforming growth factor- α (TGF- α), an EGFR ligand, in radiation-induced pulmonary fibrosis (RIPF). Preclinical studies have suggested that EGFR inhibition may prevent fibrosis (7), while activation of pneumonitis with EGFR inhibition has been reported in some lung injury models and in clinical practice (7–10). Further, although EGFR inhibition has been suggested as a method to inhibit RIPF, the specific EGFR ligands critical to the progression of RIPF remain unknown.

EGFR signaling is known to be involved in a range of pathways implicated in fibrosis, including inflammation and fibroblast proliferation (11–14). However, the role of EGFR signaling in the critical aspects of collagen elaboration and maturation has largely remained unexplored. Further, the known stimulating role of TGF- α in some fibrotic processes

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(15–17) led us to hypothesize that EGFR signaling may play a role in collagen deposition. EGFR signaling is known to activate the PI3K-Akt and MAPK pathways (18). PI3K-Akt and MAPK signaling have been shown to enhance lysyl oxidase activity, a critical mediator of collagen maturation (19–21), suggesting EGFR signaling may play a role in stimulating collagen maturation and accumulation.

In this study we explored the effects of TGF- α deficiency on the progression of radiation-induced lung injury, inflammatory cytokine expression, inflammatory infiltrates and collagen production.

METHODS AND MATERIALS

Animal Studies and Genotype Confirmation

All animal studies were institutionally approved and deemed in accordance with the guidelines of the Institute of Laboratory Animal Resources, National Research Council. C57BL6/J mice (C57-WT) and B6.129P2-Tgfa^{tm1ArJ}/J mice (TGF- $\alpha^{-/-}$) were obtained from Jackson Laboratories. Mice were bred in-house. The genotype for each subject was confirmed by PCR assay of genomic DNA isolated from tail clips (DNEasy, Qiagen, Valencia, CA) using the recommended primers and PCR conditions from Jackson Laboratories.

Female C57-WT mice, female TGF- $\alpha^{-/-}$ mice or female heterozygous progeny (TGF- $\alpha^{+/-}$) were irradiated at 8–10 weeks of age. Irradiation was performed with mice restrained in a custom Lucite jig with lead shielding that allows for selective irradiation of the thorax. Five daily fractions of 6 Gy were delivered to the thorax with a Therapix DXT300 X-ray irradiator (Pantak Inc., East Haven, CT) using 2.0 mm aluminum filtration (300 kV peak) at a dose of 2.3 Gy/min. Immediately after irradiation mice were removed from the jig and housed in a climate and light/dark controlled environment. All mice were given *ad libitum* access to food and water.

Lung tissue was collected from irradiated and control mice at 2, 4, 8 and 20 weeks after irradiation ($n \geq 3$ per condition and genotype). The right lung was snap frozen and stored at -80°C until use. The left lung was inflated with neutral buffered formalin. Formalin-fixed lung tissue was paraffin embedded and sectioned for histologic analysis. The lungs of mice euthanized because of dyspnea or found dead were inflated with neutral buffered formalin and processed for histologic analysis.

Histopathology and Histochemistry

To assess fibrotic areas, sections of lung from the 20 week time point, and sections from mice sacrificed due to illness or found dead were deparaffinized in xylene and rehydrated through a graded alcohol series to water. Sections were then incubated in Bouin's solution and then stained using Masson's trichrome with aniline blue as the collagen stain and Weigert's iron hematoxylin as the nuclear counterstain. Sections were dehydrated through graded alcohols to xylene, and coverslips were mounted with PermountTM. Stained slides were examined on a Leica DM LB2 microscope (Wetzlar, Germany), digital micrographs were captured at 40 $\times$ magnification and imported into QCapture (Quantitative Imaging Corporation, Surrey, Canada). For assessment of fibrotic areas, the percentage of area with collagen deposition was measured from five different regions in each lung using ImageJ software (open source, written by National Institutes of Health; <http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html>).

For immunohistochemical staining, sections were deparaffinized with xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed in citrate buffer, pH 6.0 (Electron Microscopy Sciences, Hatfield, PA) at 95°C for 3 min in a pressure cooker.

Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in water for 5 min. After blocking in 2.5% normal horse serum for 1 h, sections were incubated with the appropriate primary antibody for 1 h at room temperature followed by a peroxidase conjugated secondary antibody. After incubation with ImmPACT 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA), sections were washed, counterstained with hematoxylin (Sigma Aldrich, St Louis, MO), dehydrated and mounted with Permount. Antibodies against F4/80 (macrophage marker), CD3 (T-lymphocyte marker), TGF- α and lysyl oxidase (LOX) were purchased from Abcam[®] (Cambridge, MA). An antibody for neutrophil elastase (neutrophil marker) was purchased from EMD Millipore (Billerica, MA). Secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from Vector Laboratories. The number of macrophages, neutrophils or CD3 positive cells was counted on five randomly selected high power fields (40 $\times$) per group. To investigate the TGF- α levels in irradiated lungs, sections were incubated in anti-TGF- α antibody (Abcam) after blocking and treated with compatible secondary antibody conjugated to Alexa Fluor[®] 594 dye (Life Technologies, Grand Island, NY). Slides were mounted with ProLong[®] antifade reagent (Life Technologies).

Hydroxyproline Assay

The right lung ($n = 3$ mice per genotype and condition) was weighed at the time of collection, mechanically homogenized and snap frozen. Pulmonary hydroxyproline content was measured after hydrolysis of a known weight of lung tissue in 1 ml of 6 N HCl at 110°C for 18 h. Hydrosylate was analyzed with the BioVision hydroxyproline assay kit (Milpitas, CA) per manufacturer's instructions. The increase in pulmonary hydroxyproline per mouse was calculated based on total lung weight and expressed as micrograms in the lung.

For assessment of hydroxyproline content in fibroblast cultures, adherent cells were scraped into ice cold PBS and pelleted by centrifugation. Cell pellets were hydrolyzed with 6 N HCl at 110°C for 18 h and analyzed as above. Hydroxyproline content in culture supernatants was assessed after hydrolysis with an equal volume of 12 N HCl.

Enrichment Primary Pneumocytes and Fibroblasts

Primary pneumocytes were isolated from C57BL/6J mice using a modified version of a previously described method (22–24). Briefly, mice were anesthetized 10 min after intraperitoneal injection of heparin sodium (12.5 u/g body weight). Lungs were perfused with 10 ml of Hanks' balanced salt solution (HBSS) (containing 30 mM HEPES), filled with 1 mL of an enzyme cocktail [elastase 3 u/ml, 0.01% DNase I and 0.2% collagenase in HBSS containing 30 mM HEPES (Sigma Aldrich)] and incubated in 5 ml of enzyme cocktail at 37°C for 30 min. The digested tissue was carefully teased from the airways and gently swirled for 5–10 min. The resulting suspension was successively filtered through 100 μm and 40 μm FalconTM cell strainers, then centrifuged at 130g for 8 min at 4°C and resuspended in HBSS. The crude single cell suspension was applied to Ficoll density gradient isolation solution. Pneumocytes were collected from the layer of density of 1.077–1.080, washed with HBSS and then resuspended with DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (both from Life Technologies).

Primary lung fibroblasts were isolated from C57-WT mice using a previously published method with modifications (25, 26). Briefly, lungs from unirradiated C57-WT mice were digested for 45 min at 37°C in DMEM/F12 with 0.14 U/ml Liberase Blendzyme 3 (Roche Applied Science, Indianapolis, IN) and 60 U/ml DNase I (Sigma Aldrich), serially passed through 100 μm and 70 μm filters, centrifuged at 250g at 4°C and plated in tissue culture dishes in DMEM with 15% FBS. Cells were passaged when subconfluent after

harvest with trypsin-EDTA (Cellgro, Herndon, VA). All experiments were conducted with cells at passage 3 or 4.

Proliferation Assay

Enriched pneumocytes, primary fibroblasts and NIH-3T3 (ATCC®, Manassas, VA), a murine fibroblast cell line, were maintained with DMEM containing 10% FBS and 0.1% β -mercaptoethanol. NIH-3T3 and primary cells were cultured with DMEM containing 1% FBS overnight prior to use in proliferation assays. To assess the proliferation of fibroblasts and pneumocytes in response to TGF- α , 5×10^5 cells were cultured with 200 μ L complete media containing TGF- α (0.1, 1, 10 ng/mL). Cell proliferation was assessed using a BrdU cell proliferation kit (EMD Millipore) following the manufacturer's instruction.

Sirius Red Collagen Assay

A colorimetric dye-binding assay was used to assess the accumulation of soluble collagen as previously described (27). Cells were plated in RPMI with 1% FBS with 10 μ M ascorbate. Four hours after plating, cells were treated with the indicated dose of TGF- α and incubated for an additional 72 h. Collagen was measured in cell culture supernatants and in acid-pepsin treated adherent cells. Briefly, cultured cells in a 6-well plate were incubated in 500 μ L of acid-pepsin solution (0.1 mg/mL pepsin in 500 mM acetic acid) overnight at 4°C. After centrifugation (10 min at 16,000g), 100 μ L of clear supernatant from the acid-pepsin extract was incubated with 990 μ L 0.1% Sirius Red in saturated picric acid (Electron Microscopy Sciences) and agitated for 1 h at room temperature. In parallel, 100 μ L of culture supernatants were combined with 0.1% Sirius Red in saturated picric acid without acid-pepsin extraction. After centrifugation, the colored pellet was washed with 0.5 M acetic acid and dissolved in 200 μ L 0.5 M NaOH. Absorbance of samples and standards was measured at 540 nm. All collagen concentrations were normalized to total protein of the sample, as well as to the collagen concentration of the vehicle control for each specimen type.

Western Blotting

Cell and lung tissue extracts were prepared using radioimmuno-precipitation assay buffer (RIPA buffer, Pierce) containing protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich), followed by measurement of protein concentrations by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein were subjected to Western blot analysis and were probed with the following primary antibodies: ERK, pERK (Thr202/Tyr204), pAKT (Ser473), AKT, phospho mTOR (Ser2448) and mTOR were obtained (Cell Signaling Technology®); and antibodies to actin and LOX (EMD Millipore). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). ImageJ software (NIH, Bethesda, MD) was used to evaluate the relative expression of LOX normalized to actin, the loading control.

ELISA

Lung tissue was obtained from 3 mice per group at time points after irradiation as indicated. One hundred milligrams of lung tissue was homogenized in 1 mL RIPA buffer containing protease inhibitors. Soluble proteins were separated from insoluble material by centrifugation (16,000g, 10 min), and the total protein count of the resulting supernatant was determined using the Bradford method (Bio-Rad). The supernatant was then subjected to ELISA to determine the concentrations of IL1- β , IL-4, TNF- α , EGF, TGF- β , IL-6 (mouse DuoSet ELISA, R&D Systems®, Minneapolis, MN) and TGF- α (human Quantikine ELISA, R&D Systems).

NIH-3T3 cells were incubated overnight in DMEM containing 1% FBS and 0.1% β -mercaptoethanol, followed by treatment with TGF- α

(0–10 ng/mL). Culture supernatants were collected at 1, 24 and 72 h after TGF- α addition. Supernatants were subjected to ELISA to determine the concentration of TGF- β (mouse DuoSet ELISA, R&D Systems).

LOX Activity Assay

LOX enzyme activity was measured using the Fluorometric LOX activity assay kit (Abcam). Briefly, lung extracts, cell lysates or cell culture supernatants were incubated in a working solution (1:1, v/v) containing the hydrogen peroxide releasing LOX and a red fluorescent HRP substrate. After 30 min of incubation at 37°C in the dark, the reaction mixture was analyzed on a microplate reader at an excitation/emission = 540/590 nm.

Inhibitors

Beta-aminopropionitrile [(BAPN) Sigma Aldrich], a LOX inhibitor, was used at a final concentration of 7.5 μ M or 30 μ M. NIH-3T3 cells were treated with BAPN for 1 h prior to TGF- α treatment and for the duration of the experiments. A TGF- β neutralizing antibody was obtained from R&D Systems and utilized at a dose of 1 μ g/mL. TGF- α downstream pathway inhibitors, AZD6244 (Selleck Chemicals, Houston, TX), and perifosine and everolimus (both from Sigma Aldrich) were used at final concentrations of 1 μ M. Inhibitors were added 1 h prior to treatment with TGF- α and remained in the media for the duration of the experiments.

Statistical Analysis

Survival between groups was compared with the Kaplan-Meier method with unstratified log-rank statistical analysis to test for differences and pairwise comparisons between groups. For tissue studies including comparisons of strain (C57-WT vs. TGF- $\alpha^{-/-}$) and dose (0 Gy vs. 5×6 Gy), comparisons between conditions were evaluated with two-way ANOVA with Tukey correction for multiple comparisons. For *in vitro* studies, comparisons between conditions were evaluated with one-way ANOVA with Tukey correction for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant. *In vitro* studies were performed in duplicate and validated in three separate experiments. With the exception of survival, all results are reported as the mean $\pm$ standard deviation.

RESULTS

Effects of Irradiation on TGF- α Expression

To determine how radiation temporally alters levels of TGF- α in lung tissue, TGF- α protein expression was evaluated at multiple time points in lung tissue homogenates from C57-WT mice and mice deficient in TGF- α after 5×6 Gy (Fig. 1A). In C57-WT mice, lung TGF- α concentration increased dramatically at two weeks after irradiation and remained elevated for the duration of the study. As expected, no increase in TGF- α was observed in TGF- $\alpha^{-/-}$ mice.

Survival after Irradiation

To investigate the impact of TGF- α deficiency on the course of radiation lung injury, the survivals of C57-WT mice, TGF- $\alpha^{-/-}$ mice and TGF- $\alpha^{-/-}$ mice were compared after 5×6 Gy thoracic irradiation. Prior to death, irradiated mice of all genotypes experienced rapid respiration and

FIG. 1. Effects of TGF- α deficiency on radiation-induced lung injury. C57-WT and TGF- $\alpha^{-/-}$ mice were exposed to 5 × 6 Gy of thoracic irradiation. Panel A: Lung tissue was collected at the indicated time points (n = 3 mice per strain per time point) after irradiation and subjected to ELISA to determine the concentration of TGF- α . * P < 0.05 for the comparison to other strain at the same time point; points: mean, error bars: SD. Panel B: Kaplan-Meier plot of survival of irradiated mice. Panel C: Lung tissue collected at 20 weeks after irradiation from C57-WT and TGF- $\alpha^{-/-}$ mice was analyzed for hydroxyproline content (n = 3 mice per strain per time point). Columns: mean, error bars: SD. Panel D: Masson's trichrome staining of lung tissue collected at 20 weeks after irradiation. The lungs of C57-WT mice harbor extensive foci of subpleural fibrosis while those of TGF- $\alpha^{-/-}$ mice exhibit minimal fibrosis. Collagen: blue, nuclei: purple, cytoplasm/epithelia: pink. Scale bar: 50 μ m.

weight loss, suggesting a pulmonary cause of death. Necropsy revealed pale and edematous lungs in all irradiated mice, with no obvious differences noted between genotypes. In contrast, C57-WT mice and TGF- $\alpha^{+/+}$ mice were found to have small pleural effusions at necropsy while most TGF- $\alpha^{-/-}$ mice sacrificed due to dyspnea or found dead had large pulmonary effusions. These effusions were not noted in mice of any genotype or treatment group euthanized for tissue collection at 20 weeks or at any earlier time points.

The median survival of mice receiving 5 × 6 Gy irradiation was 24.4 weeks for C57-WT, 23.6 weeks for TGF- $\alpha^{+/+}$ and 48.2 weeks for TGF- $\alpha^{-/-}$ (log-rank test, P = 0.025) (Fig. 1B). Pairwise comparisons showed that C57-WT mice had inferior survival after irradiation compared to unirradiated C57-WT mice (P < 0.001) and irradiated TGF- $\alpha^{-/-}$ mice (P < 0.001). The survival of TGF- $\alpha^{+/+}$ mice exposed to 5 × 6 Gy of radiation was not statistically different than that of similarly treated C57-WT mice (pairwise comparison, P = 1.00).

Collagen Accumulation and Fibrosis

To determine if differences in survival were related to a difference in fibrosis between the strains, collagen

accumulation in the lungs of mice was assessed at 20 weeks after irradiation with assessment of hydroxyproline content and with Masson's trichrome staining (Fig. 1C, D). Hydroxyproline content in the irradiated lungs of C57-WT mice was significantly higher than that observed in lung tissue from unirradiated C57-WT mice (63.0 ± 6.9 vs. 30.5 ± 1.8 μ g per lung, P = 0.001). Although lung hydroxyproline content of irradiated TGF- $\alpha^{-/-}$ mice was significantly increased compared to unirradiated TGF- $\alpha^{-/-}$ mice (37.6 ± 5.11 vs. 22.4 ± 1.26), it was markedly reduced compared to that detected in lung tissue from irradiated C57-WT mice (37.6 ± 5.11 vs. 63.0 ± 6.9 μ g per lung, P < 0.001).

Similarly, Masson's trichrome staining of lung tissue at 20 weeks after irradiation revealed extensive collagen accumulation in the lungs of irradiated C57-WT mice, but not in the lungs of irradiated TGF- $\alpha^{-/-}$ mice (Fig. 1D). Collagen accumulation and alveolar collapse were observed to the greatest degree in the subpleural region.

To obtain information about collagen content at the time of death in C57-WT and TGF- $\alpha^{-/-}$ mice, sections of lung from mice sacrificed due to dyspnea or found dead were also stained using Masson's trichrome. Lung tissue from moribund C57-WT and TGF- $\alpha^{-/-}$ mice exhibited increased collagen at the time of death compared to the lungs of

FIG. 2. Effects of TGF- α deficiency on radiation-induced pulmonary inflammation. C57-WT and TGF- $\alpha^{-/-}$ mice were exposed to 5×6 Gy of thoracic irradiation. Lung tissue was collected at multiple time points after irradiation ($n = 3$ mice per strain per time point) and processed for immunohistochemical evaluation of macrophage (panel A), neutrophil (panel B) and T-cell (panel C) infiltration. DAB (brown) was used as the chromogen in each example (right panels) with hematoxylin as the counterstain. Columns: mean, error bars: SD, brackets: $P < 0.05$, scale bars: $12 \mu\text{m}$, high power field (HPF) ($63\times$); NE: neutrophil elastase.

unirradiated mice. However, the collagen content of irradiated TGF- $\alpha^{-/-}$ mice was intermediate between irradiated C57-WT mice and unirradiated controls (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR13625.1.S1>). Histologic examination of lung tissue from C57-WT mice and TGF- $\alpha^{-/-}$ mice that were found dead or euthanized for dyspnea revealed extensive inflammation (data not shown).

Inflammatory Infiltration

Inflammation has been implicated as a major contributor to RIPF and radiation-induced pneumonitis (28–30), with inflammatory infiltrates increasing in a time-dependent fashion after irradiation. Further, TGF- α has been shown to alter inflammatory cell recruitment (16, 31). Immunohistochemistry was used to determine if deficiency of TGF- α altered the influx of inflammatory cells after irradiation (Fig. 2). C57-WT and TGF- $\alpha^{-/-}$ mice exhibited similar increases in macrophage counts through 20 weeks after exposure to 5×6 Gy of radiation when compared to

unirradiated controls [C57-WT: 0 Gy, 15.2 ± 1.8 cells/HPF; TGF- $\alpha^{-/-}$: 0 Gy, 13.8 ± 1.5 ; C57-WT: 5×6 Gy, 26.4 ± 4.1 ; TGF- $\alpha^{-/-}$: 5×6 Gy, 23.6 ± 4.9 ($P < 0.001$ for the effect of dose and $P > 0.24$ for the effect of strain)]. Similarly, the lungs of both strains were noted to have increased neutrophil infiltration after irradiation with no evidence of strain-dependent variation in response [C57-WT: 0 Gy, 2.8 ± 1.0 cells/HPF; TGF- $\alpha^{-/-}$: 0 Gy, 2.0 ± 1.2 ; C57-WT: 5×6 Gy, 12.8 ± 2.2 ; TGF- $\alpha^{-/-}$: 5×6 Gy, 10.2 ± 1.8 ($P < 0.001$ for dose comparison and $P > 0.17$ for strain comparisons)]. No significant change in T-lymphocyte infiltration between strains was noted after irradiation (multiple comparison, $P > 0.22$).

Cytokine Production

The concentrations of the inflammatory cytokines IL-1 β , IL-4, TNF- α , TGF- β and IL-6 were evaluated in lung tissue at multiple time points after irradiation (Fig. 3). IL-1 β , IL-6 and TNF- α concentration increased from baseline as early

FIG. 3. Effects of TGF- α deficiency on radiation-induced inflammatory cytokine expression. C57-WT and TGF- $\alpha^{-/-}$ mice were exposed to 5 $\times$ 6 Gy of thoracic irradiation. Lung tissue was collected at multiple time points weeks after irradiation ($n = 3$ mice per strain per time point) and processed for ELISA. One hundred milligrams of lung was homogenized in 1 mL of lysis buffer and analyzed for the concentration of IL-1 β (panel A), IL-4 (panel B), TNF- α (panel C), IL-6 (panel D), TGF- β (panel E) and EGF (panel F). No significant differences between strains were seen for any analyte.

as the two weeks time point after irradiation, and with the greatest changes appreciated for TNF- α and IL-6. No variation in IL-4 as a function of time after irradiation was noted. At baseline there was a small but significant increase in the levels of TGF- β in the lung tissue of TGF- $\alpha^{-/-}$ mice compared to C57-WT mice. TGF- β levels increased in the lung tissue of both C57-WT mice and TGF- $\alpha^{-/-}$ mice at the two weeks time point postirradiation with a small but significant increase in the C57-WT mice relative to the TGF- $\alpha^{-/-}$ mice.

The concentration of EGF, an EGFR ligand, was similarly evaluated in lung tissue homogenates. No variation in lung EGF levels was seen at any time point in irradiated C57-WT and TGF- $\alpha^{-/-}$ lungs. Further, there was no evidence of a compensatory increase in EGF levels at baseline or after irradiation in TGF- $\alpha^{-/-}$ mice. Thus, no significant differences in the cytokine response of each strain after irradiation were appreciated.

Effects of TGF- α In Vitro

Given the lack of differences in inflammatory cytokine expression and inflammatory cell infiltration in the lungs of C57-WT and TGF- $\alpha^{-/-}$ mice after irradiation, we next sought to use *in vitro* studies to better understand the effects of TGF- α on cultured fibroblasts and pneumocytes, two lineages implicated in radiation fibrosis (1, 24). The dose of

TGF- α chosen for *in vitro* studies was based on previously published reports (32–36) in combination with our observation that TGF- α expression is heterogenous in irradiated lung (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR13625.1.S1>). *In vitro* exposure to TGF- α resulted in a dose-dependent increase in NIH-3T3 fibroblast proliferation relative to vehicle control [Fig. 4A: 0 ng/mL, 1.00 ± 0.09 ; 1 ng/mL, 1.27 ± 0.12 ; 10 ng/mL, 1.50 ± 0.09 ($P = 0.03$)]. The stimulatory effect of TGF- α on fibroblast proliferation was confirmed in NIH-3T3 cells and primary lung fibroblasts using a BrdU incorporation assay (Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR13625.1.S1>). TGF- α treatment had no significant effect on primary pneumocyte proliferation ($P = 0.15$). Treatment of NIH-3T3 fibroblasts with TGF- α resulted in an increase in mature collagen accumulation in treated cells and their secreted matrix [Fig. 4B: relative collagen, 0 ng/mL; TGF- α : 1.00 ± 0.02 ; 1 ng/mL, 1.44 ± 0.23 ; 10 ng/mL, 1.61 ± 0.12 ($P = 0.01$)], with no significant change in the amount of media-soluble, immature collagen ($P = 0.79$). These findings were confirmed with assessment of hydroxyproline content, which revealed a dose-dependent increase in mature collagen accumulation in NIH-3T3 cells and matrix after treatment with TGF- α (Fig. 4C: 0 ng/mL, TGF- α ; 25.87 ± 3.42 μ g, hydroxyproline/mL; 1 ng/mL, 36.88 ± 3.75 ; 10 ng/mL, 44.85 ± 6.00 ($P < 0.001$)). A similar effect of TGF- α treatment on primary lung fibroblast proliferation and

FIG. 4. Effects of TGF- α on proliferation and collagen production *in vitro*. NIH-3T3 cells and primary AEC were cultured in reduced serum media (1% FBS) overnight, treated with TGF- α (0, 1, 10 ng/mL) and incubated for 72 h. Panel A: Proliferation was evaluated by MTT assay and normalized to the proliferation of the vehicle treated control for each cell type. Panel B: Salt soluble collagen (no pepsin extraction) and mature collagen (cell pellet and scrapings of matrix adherent to culture plastic, with acid-pepsin extraction) production in NIH-3T3 cells were evaluated with a Sirius Red collagen assay. Values were normalized to the vehicle treated control. Panel C: Hydroxyproline content of NIH-3T3 media and cell/matrix (cell pellet and scrapings of matrix adherent to culture plastic) was assessed. Panel D: TGF- β concentration in culture supernatants was measured with ELISA at 1, 24 and 72 h after TGF- α treatment. Cell lysates were adjusted to a common protein concentration to account for differences in cell number. All *in vitro* experiments were performed in duplicate and validated in three separate experiments. Columns: mean, error bars: SD, brackets: $P < 0.05$ for comparisons between doses of TGF- α .

collagen production was observed (Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR13625.1.S1>). To exclude the possibility that TGF- α -mediated increased proliferation and collagen production were related to TGF- α -induced TGF- β secretion, TGF- β was assayed in media from NIH-3T3 cells at 72 h after treatment with TGF- α . No significant differences in TGF- β were seen at any dose of TGF- α (Fig. 4D).

Effects of TGF- α on Lysyl Oxidase

The ability of TGF- α to stimulate accumulation of mature collagen without increased levels of soluble, immature collagen suggested that TGF- α stimulation may enhance both collagen production and maturation. Lysyl oxidase (LOX), a protein involved in collagen cross linking and maturation, is known to be activated in irradiated lung tissue (37). We hypothesized that TGF- α stimulates LOX activity, thus enhancing mature collagen accumulation. Indeed, treatment of fibroblasts with TGF- α led to a dose-dependent increase in LOX expression (Fig. 5A) and LOX activity

[Fig. 5B: relative LOX activity, 0 ng/mL; TGF- α , 1.00 ± 0.58 ; 1 ng/mL, 2.18 ± 0.092 ; 10 ng/mL, 3.58 ± 0.42 ($P = 0.019$)]. Further, treatment of TGF- α stimulated NIH-3T3 cells with the LOX inhibitor BAPN resulted in a dose-dependent decrease in LOX activity and mature collagen accumulation, with the 30 μ M dose of BAPN reducing LOX activity and collagen accumulation to levels similar to untreated controls (Fig. 5C, D).

Evaluation of LOX expression in lung tissue at 20 weeks after irradiation by immunohistochemistry confirmed that LOX expression co-localized with areas of fibrosis (Fig. 6A). Assessment of LOX expression by Western blotting confirmed that radiation induces expression of LOX in C57-WT and TGF- $\alpha^{-/-}$ mice, however TGF- $\alpha^{-/-}$ mice had only a modest increase in LOX expression after irradiation (Fig. 6B, C). LOX activity increased in lung tissue of C57-WT and TGF- $\alpha^{-/-}$ mice at two weeks after exposure to 5×6 Gy of radiation but remained elevated through 20 weeks after irradiation only in C57-WT mice (Fig. 6D).

FIG. 5. Effects of TGF- α treatment on LOX activity in fibroblast cultures. NIH-3T3 cells were cultured in reduced serum media (1% FBS) overnight, treated with TGF- α (0–10 ng/mL) and incubated for 72 h. Cell pellets were collected for Western blotting analysis of LOX expression (panel A) or LOX activity (panel B). To exclude that TGF- β expression mediated the effects of TGF- α in regards to LOX activity, a TGF- β neutralizing antibody (1 μ g/mL) was added to NIH-3T3 cells 1 h prior to TGF- α treatment (0–10 ng/mL), and LOX inhibitor BAPN (7.5 μ M) was used for negative control. To confirm the importance of LOX in TGF- α mediated collagen accumulation, the LOX inhibitor BAPN (7.5 and 30 μ M) was added to NIH-3T3 cells 1 h prior to TGF- α treatment (10 ng/mL). The LOX inhibitor BAPN was added to NIH-3T3 cells in varying concentrations 1 h prior to TGF- α or vehicle treatment (10 μ M). Levels of LOX activity (panel C) and mature collagen (panel D) were assessed at 72 h. Hydroxyproline content was assessed after acid-pepsin extraction of the cells and matrix adherent to the tissue culture plastic and was normalized to the total protein content of each sample. Relative values of LOX activity were normalized to vehicle treated control. Cell lysate volumes were adjusted to a common protein concentration to account for differences in cell number. All *in vitro* experiments were performed in duplicate and validated in three separate experiments. Columns: mean, error bars: SD; * P < 0.05 for the comparison to vehicle at the same dose of TGF- α , brackets: P < 0.05 for the comparison.

Effects of TGF- α Signaling on LOX Activity

TGF- α signals through EGFR and downstream intermediates. To elucidate the signaling pathway responsible for increased LOX activity after TGF- α stimulation, NIH-3T3 cells were treated with TGF- α in the presence of a MEK inhibitor (AZD6244), an Akt/PI3K inhibitor (perifosine) and an mTOR inhibitor (everolimus). Treatment with perifosine or everolimus reduced LOX expression after TGF- α stimulation, whereas MEK inhibition had minimal effects (Fig. 7A). Similarly, both everolimus and perifosine treatment reduced LOX activity

and collagen production in TGF- α -treated NIH-3T3 cells (Fig. 7C, D).

DISCUSSION

Pulmonary injury from radiation can lead to progressive fibrosis. In the current study, we examined the importance of TGF- α signaling in RIPF, and observed that TGF- $\alpha^{-/-}$ mice are highly resistant to pulmonary irradiation compared to C57-WT mice. This resistance required complete deficiency of TGF- α , as evidenced by the similar survival

FIG. 6. Effects of TGF- α deficiency on LOX expression and activity in murine lungs. C57-WT mice and TGF- $\alpha^{-/-}$ mice were exposed to 5 $\times$ 6 Gy of thoracic irradiation. Lung tissue ($n = 3$ mice per strain and treatment) was collected at 20 weeks after irradiation and LOX expression was assessed by (panel A) immunohistochemistry or (panel B) Western blotting. Panel C: Densitometric analysis of Western blotting ($n = 5$ mice per strain and treatment) for LOX expression. Panel D: Lung tissue from C57-WT mice and TGF- $\alpha^{-/-}$ mice was collected at multiple time points after irradiation ($n = 3$ per strain and time point), homogenized and assessed for LOX activity. Columns: mean, error bars: SD; brackets: $P < 0.05$; points: mean, $*P < 0.05$ for the comparison between strains at the time point.

of TGF- $\alpha^{+/+}$ and C57-WT mice. Combined with the finding that the lungs of TGF- $\alpha^{-/-}$ mice had far less collagen accumulation compared to C57-WT mice, these findings confirm that TGF- α deficiency protects from RIPF.

TGF- α was found to be rapidly expressed in the lungs of C57-WT mice after irradiation and remained elevated through the 20 weeks time point. This finding is consistent with previous reports of TGF- α expression in pulmonary fibroblasts exposed to oxidant stress (17). We next investigated the possible mechanisms by which TGF- α deficiency prevents RIPF by examining proinflammatory cytokine expression and influx of inflammatory cells. Our results revealed no significant change in the expression of proinflammatory cytokines, suggesting that a reduction in inflammation was not the primary mechanism behind the resistance of TGF- $\alpha^{-/-}$ mice to irradiation. Indeed, a lack of

effect of TGF- α deficiency on inflammation was confirmed by examining infiltrating immune cells in the lungs of irradiated mice. We observed no significant differences between C57-WT and TGF- $\alpha^{-/-}$ mice in the type or number of macrophages, neutrophils or T-lymphocytes at baseline or after irradiation. Chronic inflammation has been described as a driver of RIPF (38), however the data presented here suggest that deficiency of TGF- $\alpha^{-/-}$ can reduce collagen accumulation and fibrosis, even in the setting of unabated chronic inflammation.

Previously, inducible TGF- α expression in lungs has been shown to lead to progressive fibrosis (39), which can be blocked with EGFR inhibition (40), however the mechanism of this effect has remained elusive (15). To better understand if TGF- α plays a direct role in stimulating fibrosis, we performed *in vitro* experiments with murine

FIG. 7. The effect of down-stream inhibitors on TGF- α mediated profibrotic signaling. NIH-3T3 cells were cultured in reduced serum media (1% FBS) overnight and treated with the indicated inhibitor in DMSO (1 μ M) or DMSO as a vehicle control. One hour after the addition of the inhibitors, TGF- α (0 or 10 ng/mL) was added. Cell pellets and cell culture supernatants were collected 72 h after the addition of TGF- α . Cell lysate volumes were adjusted to a common protein concentration to account for differences in cell number. Media was analyzed without dilution. Panel A: LOX expression was evaluated by Western blotting. Panel B: LOX activity was assessed in NIH-3T3 cell culture supernatants and cell lysates. Panel C: Media soluble collagen (no pepsin extraction) and mature collagen (cell pellet and scrapings of matrix adherent to culture plastic, with acid-pepsin extraction) production in NIH-3T3 cells were evaluated with a Sirius Red collagen assay. Values were normalized to the vehicle treated control. Panel D: Hydroxyproline content of NIH-3T3 media and cell/matrix (cell pellet and scrapings of matrix adherent to culture plastic) was assessed. Values were normalized to total protein in each sample. All *in vitro* experiments were performed in duplicate and validated in three separate experiments. Columns: mean, error bars: SD, brackets: $P < 0.05$ for the comparison.

fibroblasts. We confirmed the previously published findings that treatment with TGF- α stimulates fibroblast proliferation (32, 41). We also observed a novel finding that TGF- α stimulated the production of mature collagen, whereas production of soluble collagen was not altered. This led us to further investigate the effects of TGF- α on LOX, an enzyme involved in the final steps in collagen maturation.

Previously, LOX activity has been shown to be regulated by TGF- β (42–44) and indirectly by TNF- α through TGF- β (20). TNF- α levels were similar between TGF- $\alpha^{-/-}$ and C57-WT mice at all time points, suggesting that a reduction in TNF- α was not responsible for the reduction in LOX expression and activity in TGF- $\alpha^{-/-}$ compared to C57-WT mice. TGF- β was elevated after irradiation in both C57-WT and TGF- $\alpha^{-/-}$ mice. Although it is possible that the increase in LOX activity and expression in our animal model was due to an increase in TGF- β expression, the similar extent of

TGF- β elevation between the two strains and the persistent elevation of LOX activity through 20 weeks *in vivo* in the absence of corresponding elevation in TGF- β levels suggests that another process contributes to the increase in LOX expression and activity.

The treatment of fibroblasts *in vitro* with TGF- α was sufficient to increase LOX activity and expression with no measurable change in TGF- β expression, suggesting a direct effect of TGF- α signaling on LOX expression and activity. The importance of LOX activation in the accumulation of mature collagen after TGF- α exposure was confirmed by the ability of a LOX inhibitor to prevent the increase in collagen accumulation observed after TGF- α treatment in fibroblasts. *In vivo*, TGF- $\alpha^{-/-}$ mice experienced a peak in LOX activity at 2 weeks, similar to C57-WT mice. However, LOX activity in TGF- $\alpha^{-/-}$ mice returned to near baseline levels at 4 weeks after irradiation and remained lower than that of C57-WT

mice at the later time points. We hypothesize that transient elevations in TGF- β and TNF- α after radiation-induced LOX activity in TGF- $\alpha^{-/-}$ mice but were insufficient to sustain chronic elevation. Conversely, TGF- α remained persistently elevated in C57-WT mice and may be responsible for ongoing stimulation of LOX activity in this model.

To elucidate the downstream pathways responsible for LOX activation by TGF- α , NIH-3T3 cells were treated with inhibitors of signaling downstream of EGFR. Both everolimus (mTOR inhibitor) and perifosine (AKT/PI3K inhibitor) treatment were capable of inhibiting LOX activation and collagen expression in TGF- α treated fibroblasts. These findings support the importance of Akt and mTOR signaling in the profibrotic effects of TGF- α . No such benefit was observed with MEK inhibition, another pathway known to be activated by TGF- α signaling. Indeed, the mTOR pathway is a critical signaling pathway in the pulmonary fibrosis induced by targeted pulmonary TGF- α expression (45). Our data suggests that at least part of the beneficial effect of mTOR inhibition in this setting may be due to inhibition of LOX expression and activity. Further, mTOR inhibition may provide a viable opportunity to target LOX activation after irradiation.

The duration of TGF- α induction in the lungs of irradiated mice supports the possibility that TGF- α may continue to modulate the pulmonary environment for prolonged periods after fibrogenic irradiation. The lack of induction of EGF expression suggests that while EGFR may play a critical role in the radiation response of lungs, EGF does not. Indeed, a number of studies have shown an increase in expression or phosphorylation of EGFR after radiation exposure in lung or in the setting of lung fibrosis from a variety of causes (6, 7, 46). However, EGF expression was not reported in these studies. Collectively, these findings suggest that the negative consequences of EGFR activation after irradiation with regard to pulmonary fibrosis are due to TGF- α , not EGF.

There is conflicting literature regarding the effects of EGFR inhibition after lung irradiation. For example, delivery of gefitinib, an EGFR inhibitor, after irradiation in rats has been shown to increase inflammation but decrease fibrosis (7). This is consistent with the clinical literature that reports activation of radiation-induced pneumonitis with EGFR inhibitor therapy (47). A second study found that gefitinib delivered in the acute phase had no effect on the rates of fibrosis after irradiation, but delivery during the chronic phase markedly reduced fibrosis (46). In our study, absence of TGF- α did not enhance inflammation or the expression of proinflammatory cytokines, suggesting that the effects of gefitinib on inflammation are due to inhibition of EGFR activation by other endogenous ligands or from off-target effects. Indeed, EGF is known to regulate inflammatory gene expression and inflammation (48). These findings raise the possibility that different EGFR ligands may play divergent roles in radiation-induced lung injury, and that targeting a specific

ligand, such as TGF- α , instead of the receptor may allow modulation of fibrosis without the concern for activation of inflammation. Certainly, clarifying this relationship will require further work, however, understanding the molecular pathways underlying this injury may provide additional therapeutic opportunities.

Although it is likely that rodent models may not completely recapitulate the human condition, studying these effects in human patients is complicated by a number of issues. Obtaining tissue from fibrotic lung after radiotherapy in patients is rarely possible. In addition, the risk of pulmonary injury after radiation exposure can be altered by many confounding factors such as the presence of tumor, differences in dose and volume of exposure between patients and the varying extent of comorbidities that may alter susceptibility to radiation-induced lung injury. Thus, the use of animal models has been a necessary tool to develop hypotheses about the causes and possible treatments of radiation-induced pulmonary fibrosis in human patients.

In summary, we identify TGF- α as a critical molecule in the setting of radiation-induced fibrosis. Expression of TGF- α is rapid and prolonged in lung tissues after irradiation. TGF- α is capable of stimulating LOX activity and in turn the production of mature collagen, a hallmark of fibrosis.

SUPPLEMENTARY INFORMATION

Supplementary Fig. S1. C57-WT mice were exposed to 5×6 Gy of thoracic irradiation. Lung tissue was collected at two weeks after irradiation. Immunofluorescence for TGF- α was performed to determine the pattern of expression in irradiated and unirradiated lung. Panel A: Localized TGF- α expression was observed in lung parenchyma in a patchy distribution. Panel B: Intense regions of TGF- α expression were localized to the subpleural zone of irradiated lungs. A white dashed line has been added to the photograph to localize the pleural surface, with lung parenchyma located to the left of this line.

Supplementary Fig. S2. Primary lung fibroblasts (panel A) and NIH-3T3 cells (panel B) were cultured in reduced serum media (1% FBS) overnight, treated with TGF- α (0–10 ng/ml) and incubated for 72 h. BrdU incorporation during the last 4 h of the 72 h incubation was measured. Panel C: Primary lung fibroblasts isolated from C57-WT mice were cultured in reduced serum media (1% FBS) overnight, treated with TGF- α (0–10 ng/mL) and incubated for 72 h. Cell pellets were collected at 72 h and analyzed for hydroxyproline concentration. All *in vitro* experiments were performed in duplicate and validated in three separate experiments. Columns: mean, error bars: SD, * $P < 0.05$ for comparison to 0 ng/ml TGF- α .

Supplementary Fig. S3. NIH-3T3 cells were cultured in reduced serum media (1% FBS) overnight and treated with

TGF- α (0, 1 or 10 ng/mL). Cell pellets were collected 1 h after the addition of TGF- α . Cell lysates were subjected to Western blot analysis with actin as a loading control.

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