Attenuated Lung Fibrosis in Interleukin 6 Knock-out Mice after C-ion Irradiation to Lung

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INTRODUCTION

Lung carcinoma is the leading cause of cancer mortality in industrialized countries.1,2) Radiotherapy (RT) is the most important treatment modality for inoperable, locally advanced non-small-cell lung cancer (NSCLC), but conventional RT with doses of 60–70 Gy is often insufficient to eradicate the tumor, with local control rates of only 10–20% at one year following irradiation.3,4) RT dose escalation is a promising approach, although it may be limited by the low tolerance of surrounding normal lung tissue. Radiation pneumonitis and radiation fibrosis, which represent the acute and late phase of radiation-induced lung injury, respectively, are the major dose-limiting toxicities due to RT for NSCLC. About 10–15% of NSCLC patients develop severe lung toxicity after thoracic irradiation, and a notable percentage of these patients die from radiation pneumonitis.4,5)

A carbon-ion (C-ion) beam offers high-linear energy transfer and other advantages including high relative biologic effectiveness (RBE), oxygen- or cell-cycle-independent cell damage, and a high rate of double-strand DNA breaks.6,7) Indeed, the local control rate for 50 patients with Stage I NSCLC using a hypofractionated regimen reached 94.7%.8) Such high-rate and definite tumor control is an outstanding feature of C-ion RT that could result predominantly from the radiobiological nature of the high-energy transfer beams and may contribute to improved survival for patients with Stage I NSCLC. Lung is a dose-limiting organ for RT, including C-ion RT of cancer in the thoracic region;9) however, the biological mechanisms underlying the action of C-ion irradiation remain largely unknown. Animal studies showed that the C57BL/6J (WT) mouse strain is prone to lung fibrosis after thoracic irradiation with X-rays and gamma-rays,10,11) but no studies have reported the responses to C-ion RT.

Inflammatory cytokines and various immune cells are associated with the inflammation-related effects of pneumonitis and fibrosis following lung irradiation.12,13) Interleukin (IL)-6 is a proinflammatory cytokine that mediates many inflammatory processes in the lung, and the dysregulated release of IL-6 has been implicated in the pathogenesis of a variety of respiratory disorders.14,15) Clinical studies also

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suggested that changes in the plasma levels of IL-6 during RT could be used to identify patients at risk of radiation-induced pneumonitis and fibrosis.18–20 These reports indicated that IL-6 contributes to the development and maintenance of lung fibrosis.

We previously reported that IL-6 knockout (KO) mice show radioresistance in the acute phase of alveolar damage after X-ray irradiation.21 No acute inflammation responses were apparent in histological analyses of either WT or IL-6 KO mice after thoracic irradiation. The number of CD44-positive cells in the lungs of WT mice increased within 1 hour after irradiation and then decreased at 12 hours. Similarly, the number of Bak-positive cells increased within 12 hours after irradiation and then decreased at 48–72 hours in the WT lungs. These time-dependent changes in CD44 and Bak were not observed in the lungs of IL-6 KO mice. CD44 plays an important role in the clearance of hyaluronic acid (HA), which accumulates at sites of inflammation,22,23 and Bak is a sensitive marker for apoptotic cells. Therefore, our previous data suggested that long-term lung injury after irradiation might be prevented in mice with suppressed IL-6 expression. Another group also demonstrated that lung fibrosis induced by bleomycin (BLM) was attenuated in IL-6 KO mice.24 In that study, the KO mice showed significantly less BLM-induced inflammatory cell accumulation and subsequent fibrotic changes, and decreased expression of fibrosis-related mediators including transforming growth factor-β1 (TGF-β1) and CC chemokine ligand 3 (CCL3). These results supported a critical role for IL-6 in the development of pulmonary inflammatory responses and fibrosis due to BLM.

In this study, we performed whole-lung C-ion irradiation on IL-6 KO mice to investigate the role of IL-6 during the late phase of radiation-induced lung inflammation. We found that lung fibrosis was attenuated in IL-6 KO mice after C-ion irradiation with 10 Gy. To evaluate the chronic phase that is characterized by lung fibrosis, we examined pathological changes and macrophage infiltration in lung tissue obtained 12 and 24 weeks after C-ion thoracic irradiation.

**MATERIALS AND METHODS**

**Mice**

The IL-6 KO mice were a kind gift of Dr. Iwakura, and were bred in the National Institute of Radiological Sciences (NIRS). The IL-6 gene was disrupted in the second exon (first coding exon) by insertion of a neo cassette. F1 mice (C57BL/6 × 129 Sv) heterozygous for the mutation (IL-6+/−) were interbred to obtain homozygous (IL-6−/−). C57BL/6JSc mice (WT mice) were obtained commercially (Japan SLC, Shizuoka, Japan) as reference mice. All mice were housed and maintained in the mouse colony of the NIRS with a maximum of five mice per cage. All experiments were performed on female mice at 8 to 9 weeks old. The scheduling was designed to assess fibrosis in the lung tissue of IL-6 KO and WT mice at 12 and 24 weeks after lung irradiation. The study protocol was reviewed and approved by the NIRS Institutional Animal Care and Use Committee (protocol number 07-2014-4).

**Irradiation**

C-ion irradiation (290 MeV/n, 6 cm spread-out Bragg peak) was applied in a single dose as described previously.26 After anesthesia with pentobarbital (50 mg/kg body weight, intraperitoneal injection), the whole thorax was locally irradiated at 10 Gy with C-ion beams generated by the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the NIRS. The absorbed dose was adjusted at the center of the thorax, which was 7 mm downstream from the entrance of the skin. The control mice received anesthesia, but no irradiation.

**Tissue isolation and histological examination**

Three mice of each strain were sacrificed for lung histological analysis. After anesthesia, the left lungs of the mice were perfused in situ via the trachea with 10% neutral-buffered formaldehyde; the lungs were subsequently removed and placed in fixative as described previously.24 The fixed lung tissue was processed into paraffin and sections of 3 μm in thickness were prepared for analysis from the midhorizontal sections of the lung lobes encompassing the largest surface area. Sections were stained by hematoxylin and eosin (H&E) to examine general tissue morphology, and by Masson’s trichrome staining to visualize the connective tissue (collagen fibers).

**Immunohistochemistry**

All tissue sections were stained using a Discovery XT automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s instructions. Heat-induced epitope retrieval was performed and a standard DAB detection kit was used for visualization of the immunoreaction (Ventana Medical Systems). IL-6 expression was detected using a goat anti-mouse IL-6 polyclonal antibody (sc1265; Santa Cruz Biotechnology) diluted 1:100. Mac3 expression was detected using a rat anti-mouse Mac3 monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ) diluted 1:100. Sections were incubated for 40 min at 37°C with the primary antibodies and for 40 min at 37°C with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA) as secondary antibodies for IL-6, and biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) as secondary antibodies for Mac3. Negative controls subjected to the same procedure without the primary antibody step showed consistently negative results.

**Measurement of immunoreactivity for IL-6**

IL-6 immunoreactivity was evaluated by two observers in a coded manner without knowledge of the tissue source.
(irradiated or nonirradiated). The immunoreactivity was quantified by computerized image analysis as previously described.\(^{27}\) To ensure objectivity, eight representative, non-contiguous, nonoverlapping fields (bronchiolar epithelium not included) were systematically selected and analyzed in each slide (\(\times 40\) objective), and then analyzed using the WinROOF analysis system (Mitani, Fukui, Japan) with macroinstructions. Analysis was performed after transformation of color information to hue-saturation-intensity information. After evaluating several fields on the positive control slides, an intensity threshold for IL-6 staining was set. The IL-6 expression was quantified as the percentage positively stained area relative to the total lung tissue area.

**Measurement of Masson’s trichrome staining levels in the lung**

Masson’s trichrome staining was evaluated by two observers in a coded manner without knowledge of the tissue source (IL-6 KO or WT mice). The magnitude of blue staining in lung cells was measured by computerized quantitative image analysis as previously described.\(^{28}\) Briefly, two fields imaged at \(\times 200\) magnification in the apical areas were captured as digital images, and then analyzed using the WinROOF analysis system with macroinstructions. Analysis was performed after transformation of color information to hue-saturation-intensity information. After evaluating several fields on the positive control slides, an intensity threshold for the collagen staining (blue) was set. The proportion of stained area relative to the total area of lung cells was

![Fig. 1. H&E staining of lung tissues. (A) Original magnification, \(\times 40\). The WT mice showed fibrotic changes (WT 10 Gy 24 w; arrowheads) but not IL-6 KO mice. (B) Original magnification, \(\times 200\) in left images and \(\times 400\) in right images at 10 Gy 24 w. The WT mice showed thickening of the bronchiolar (left image; arrowhead) and alveolar walls (right image; arrows), while no such change was observed in the IL-6 KO mice.](image-url)
defined as the blue area (%).

Statistical analysis

The Mann-Whitney U test was used to analyze differences between groups. A $P$ value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Fibrotic changes and IL-6 expression induced by C-ion beam irradiation

Mild fibrotic loci were observed in the peripheral lobes of C57BL/6J mice (WT mice) lungs at 24 weeks after C-ion beam irradiation, but not at 12 weeks (Fig. 1). The WT mice also showed thickening of the bronchiolar and alveolar walls at 24 weeks after irradiation; the IL-6 KO mice did not show these changes (Fig. 1B). Next, we analyzed whether IL-6 expression is induced in WT mice by C-ion irradiation.

A time course of IL-6 immunoreactivity in the lung after irradiation with 10 Gy showed low levels in nonirradiated lung tissue of WT mice (Fig. 2). Parts of the bronchiolar epithelium, blood vessels (data not shown) and a few alveolar monocytes were immunopositive for IL-6 in these lung sections (Fig. 2A and B). The bronchiolar epithelium and monocytes in the alveolar septa and alveolar spaces were intensely positive for IL-6 at 12 and 24 weeks after irradiation (Fig. 2A), with pneumocytes and fibroblast cells showing slight immunoreactivity at 12 and 24 weeks after irradiation (Fig. 2B).

The IL-6 expression sites in nonirradiated and radiated

![Image](https://via.placeholder.com/150)

**Fig. 2.** IL-6 immunostaining of lung tissues in C57BL/6J (WT) mice. (A) Original magnification, ×400. In parts of the bronchiolar epithelium, IL-6 expression was induced intensely at 12 and 24 weeks after irradiation. (B) Original magnification, ×1000. Pneumocytes, parts of monocytes, and fibroblast cells showed slightly increased staining for IL-6 at 12 and 24 weeks after irradiation. The graph is a quantification of immunoreactivity for IL-6 in pneumocytes, monocytes, and fibroblast cells of WT mice. Staining area/total area (%) was evaluated in sham-irradiated and irradiated lung tissue of WT mice. Eight images per mouse (n = 3–5) were captured for this analysis. All results were expressed as mean +/- SD. (*indicates $P < 0.01$).
tissue observed in this study are consistent with the findings of Rube et al. using X-ray irradiation with 12 Gy,27) with the exception of the fibroblasts and blood vessels. However, these other authors reported an increase in radiation-induced IL-6 expression at 12 h and 8 weeks after irradiation that decreased back to the control level (nonirradiation) at 24 weeks after irradiation. Although it remains to be determined whether this difference in the time course for IL-6 expression between X-ray and C-ion irradiation depends on the dose or kind of radiation, it suggests that the induction of IL-6 expression by C-ion irradiation exerts fibrotic effects on lung.

Increased IL-6 levels have been reported in lung inflammation, with lung fibroblasts identified as a source of this cytokine. Indeed, IL-6 was shown to have an anti-apoptotic and proliferative effect in cultured human lung fibroblast cells in the pathological status.29–32) Lung irradiation induces an immediate epithelial reaction, with the bronchiolar epithelium becoming a significant source of IL-6 capable of promoting inflammation through recruitment and activation of inflammatory cells.27) The thickening of bronchiolar and alveolar walls in WT mice in this study, and the absence of such an effect in the IL-6 KO mice, thus implicated IL-6 production from bronchiolar epithelium, fibroblasts, pneumocyte and monocytes induced by the irradiation in the subsequent inflammatory reaction.

**Macrophage infiltration into alveolar space**

Infiltration of monocytes into the alveolar space was observed in both WT mice and IL-6 KO mice (Fig. 3). Immunohistochemical staining of the mice lung tissue using an antibody against the macrophage marker, Mac3, localized macrophages to the alveolar septa in WT and IL-6 KO mice following sham irradiation and at 12 weeks after irradiation (Fig. 3).

Macrophages are a heterogeneous population of cells made up of type-1 and type-2 subsets. Type-1 macrophages (M1) secrete proinflammatory cytokines such as IL-6, IL-1β, and TNFα, while Type-2 macrophages (M2) predominantly secrete IL-10.33) Trujillo et al.34) reported that lung injury attributable to the intrapulmonary introduction of BLM is mediated, in part, via M1 macrophages. While the infiltration of alveolar macrophages in this study was not due to IL-6 expression, the irradiation might have changed the macrophage phenotype into the M1 subtype, thus implicating IL-6 from M1 cells in the lung fibrotic changes in WT mice.

**Collagen deposition by Masson's trichrome staining**

The Masson’s trichrome staining of lungs from sham-irradiated controls and at 12 weeks after irradiation in WT mice was comparable with that in the IL-6 KO mice, showing no pathological effects. At 24 weeks after irradiation, the lungs of WT mice showed collagen deposition (Fig. 4). The collagen deposition and infiltration seemed most apparent in the apical and peripheral alveolar cells and the structure of alveolar septa was severely disrupted. In IL-6 KO mice, the collagen deposition and infiltration was attenuated at 24 weeks after irradiation. Transgenic TGF-β1 (TG) mice revealed increased collagen fibers, particularly in perivascular and peribronchial areas, but also in the alveolar walls.35) In this study, such a collagen increase was observed in either perivascular or peribronchial areas in both WT and IL-6 KO mice at 24 weeks after irradiation; however, there was no difference in these areas between WT and IL-6 KO mice (Fig. 4).

![Fig. 3. Mac3 immunostaining of lung tissues. Original magnification, ×400. The images on the right, taken at 24 weeks after irradiation show the macrophage infiltration of alveolar spaces in both WT and IL-6 KO lung (WT 10 Gy 24 w and KO 10 Gy 24 w).](image-url)
Fibrotic changes to the mice lungs were assessed based on the blue-stained areas in apical areas. Two images from apical areas of the lung at 24 weeks after irradiation were selected per tissue section for the analysis, and the blue staining was quantified for intensity using a computerized image analysis system (Fig. 5A and B). The percentage of the blue area was significantly lower in IL-6 KO mice compared to that in WT mice at 24 weeks after irradiation (Fig. 5C). These results indicated that irradiation-induced lung fibrosis was largely absent in the IL-6 KO mice.

The experiments thus far demonstrated the contribution of IL-6 to the development of C-ion irradiation-induced lung
fibrosis using IL-6 KO mice. Saito et al.\(^2\) showed that IL-6 KO mice treated with BLM had significantly suppressed TGF-β1 expression and fibrosis. In another report, TGF-β1 expression was reduced by recombinant IL-6 treatment in skin and dermal fibroblasts from IL-6 KO mice.\(^3\) Although it remains to be determined whether IL-6 acts on fibrocytes via upregulation of TGF-β1 or other inflammatory mediator, these results including ours confirm that IL-6 plays a key role in radiation-induced fibrosis.

This study is the second to use IL-6 KO mice with pulmonary irradiation. Our results indicated a difference in the fibrotic changes induced in lung tissue by irradiation between IL-6 KO and WT mice; however, we did not observe significant differences in acute inflammation between them at 4 weeks.\(^4\) Shannon et al.\(^5\) also used IL-6 KO mice to show that suppressed IL-6 expression does not reduce the degree of the subsequent acute inflammatory response after lung vascular injury. These evidences don’t mean that IL-6 does not play an important role in biological systems. IL-6 might be more important in the chronic pathological state rather than in acute inflammatory responses. Various cytokines initiate and sustain the inflammatory and fibrogenic processes associated with radiation-induced lung injury. Thus, IL-6 KO mice was useful for analyzing the function of IL-6 in this study, which examined the effect of loss of function in the long term.

In conclusion, we presented a role for the proinflammatory cytokine IL-6 in radiation-induced lung morbidity during the late phase of the inflammatory response. IL-6 KO mice showed attenuated pulmonary fibrotic changes compared to WT mice that developed mild fibrosis in peripheral lung lobes after C-ion thoracic irradiation with 10 Gy. Comparative histological examination for irradiated lungs between IL-6 KO mice and WT mice suggested proliferation of the bronchiolar and alveolar epithelia induced by the production of IL-6 following the lung irradiation.

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