Suplatast tosilate reduces radiation-induced lung injury in mice through suppression of oxidative stress

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Funding: This study was partially supported by a grant from Ryokufukai, Hiroshima University. The funding source had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Declarations of interest: none

Abstract

Purpose: Although radiotherapy is important in the treatment of malignant thoracic tumors, it has harmful effects on healthy tissues. We previously showed that suplatast tosilate, an anti-allergic agent, scavenged reactive oxygen species (ROS), including hydroxyl radicals. Because ROS-mediated oxidative stress is involved in radiation-induced lung injury, we hypothesized that suplatast tosilate could reduce radiation-induced lung injury via suppression of oxidative stress.

Methods and Materials: Murine alveolar epithelial cells were irradiated with or without a medium containing suplatast tosilate *in vitro* to determine whether the agent had cytoprotective effects against radiation-induced injury. On the other hand, the thoracic region of C57BL/6 mice was exposed to a single irradiation dose of 15 Gy and the effects of suplatast tosilate were determined by a histological evaluation and assessment of the following parameters: cell number and inflammatory cytokine levels in bronchoalveolar lavage fluid, and oxidative stress markers and hydroxyproline content in pulmonary tissues.

Results: Suplatast tosilate protected murine alveolar epithelial cells *in vitro* from irradiation-induced inhibition of cell proliferation, which was accompanied by the suppression of intracellular ROS and DNA double-strand breaks induced by irradiation. Oxidative stress markers and the levels of inflammatory and fibrogenic cytokines were upregulated in irradiated murine lungs *in vivo*. Suplatast tosilate suppressed both oxidative stress markers and the levels of cytokines, which resulted in reduced pulmonary fibrosis and clearly improved the survival rate after irradiation.

Conclusions: These findings demonstrate that suplatast tosilate could be a useful lung-protective agent that acts via suppression of oxidative stress associated with thoracic radiotherapy.

Key Words

Oxidative stress, Pulmonary fibrosis, Radiation-induced lung injury, Reactive oxygen species, Suplatast tosilate

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Abbreviations: ROS: reactive oxygen species; RT: radiotherapy; DMEM: Dulbecco's Modified Eagle Medium; DSB: DNA double-strand break; ST: suplatast tosilate; PBS: phosphate-buffered saline; BALF: bronchoalveolar lavage fluid; SOD: superoxide dismutase; ELISA: enzyme-linked immunosorbent assay; H&E: hematoxylin and eosin; BAL: bronchoalveolar lavage

1. Introduction

Radiotherapy (RT) plays important roles in the treatment of thoracic malignant tumors; however, it not only has antitumor effects but also has harmful effects on healthy tissues. These harmful effects lead to RT dose limitation. Radiation-induced lung injury, usually observed several months after irradiation, involves radiation pneumonitis and subsequent chronic pulmonary fibrosis. Radiation-induced pulmonary fibrosis is considered a result of repair mechanisms after radiation pneumonitis.[1] The frequency and severity of radiation pneumonitis depend on various risk factors including radiation dose, irradiated area, number of irradiations, the interval between irradiations, and use or non-use of concomitant chemotherapy.[2] Patients with subacute, progressive, and symptomatic radiation pneumonitis are generally treated with corticosteroids, although there is little evidence for their efficacy. Prolonged severe radiation pneumonitis results in respiratory failure, irreversible pulmonary fibrosis, and impaired quality of life.[3] Therefore, development of reliable preventive and therapeutic methods is urgently required. Radiation-induced lung injury is linked not only to direct DNA damage caused by radiation but also to indirect damage caused by reactive oxygen species (ROS) generated from water molecules in tissues. Approximately 60% to 70% of radiation-induced injury has been reported to develop due to ROS, and ROS-targeting treatments have been found effective in animal models of radiation-induced lung injury.[4,5] To date, however, no ROS-targeting treatment is clinically available for the treatment of radiation-induced lung injury.

Suplatast tosilate, an anti-allergic agent, is widely used for the treatment of bronchial asthma, atopic dermatitis, and allergic rhinitis in Japan. When suplatast tosilate is orally administered, it is absorbed from the intestinal tract and partially decomposed into 4-acrylanilide after the removal of dimethyl sulfide.[6] These compounds are considered to be the main active constituents of suplatast tosilate. The effects of suplatast tosilate have been reported to depend on the suppression of (i) eosinophil infiltration via the suppression of IL-4 and IL-5 production by helper T-cells, (ii) release of chemical mediators from adipocytes, and (iii) IgE production. Thus suplatast tosilate is considered a Th2 cytokine inhibitor.[7–9] In our previous study, however, suplatast tosilate suppressed pulmonary fibrosis in a murine model of bleomycin-induced pulmonary fibrosis independently of Th2 cytokine inhibition.[10] In addition, it protected against hyperoxic lung injury by scavenging ROS.[11] As ROS are also involved in bleomycin-induced pulmonary fibrosis, the suppressive effect of suplatast tosilate on bleomycin-induced pulmonary fibrosis might be attributable to antioxidant effects via scavenging of ROS.[12,13]

On the basis of these findings, we hypothesized that suplatast tosilate could alleviate radiation-induced lung injury via antioxidant effects. To test this hypothesis, we first determined whether suplatast tosilate could protect murine alveolar epithelial cells against radiation-induced injury *in vitro*. We further assessed the effects of suplatast tosilate in a murine model of radiation-induced lung injury along with its potential to affect the radiation sensitivity of tumors *in vivo*.

2. Methods and Materials

2.1. Cells and cell culture

Murine alveolar epithelial cells (LA-4) and Lewis lung carcinoma cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and American Type Culture Collection (Manassas, VA), respectively. These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37 °C in a 5% CO_2 incubator.

2.2. Animals

Specific pathogen-free female C57BL/6 mice (8–10 weeks old) were purchased from Charles River Laboratories Japan (Yokohama, Japan). The animals were housed in pathogen-free rooms with a controlled environment under a 12-h light-dark cycle and maintained on laboratory chow, with free access to food and water. The committee on animal research at Hiroshima University approved all procedures (approved no. A15-64).

2.3. Cell irradiation and clonogenic assay

LA-4 cells were seeded at a density of 5000 cells/well in 24-well plates. After 24 h, suplatast tosilate (Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) was added to the medium at final concentrations of 0 and 50 μg/mL (100 μM) for 2 h. Thereafter, the cells were further exposed to a single dose of 5 Gy of irradiation using an X-ray generator (MBR-1520; Hitachi, Tokyo, Japan) at 150 kVp and 20 mA, with a filter of 0.5 mm aluminum and 0.1 mm copper. The dose rate was approximately 5.0–5.2 Gy/min. After 24 h, cells were trypsinized, counted, and seeded in new 6-well plates containing nutrient medium at a density expected to yield ~100 colonies per dish. The dishes were then incubated for 10 days. The resultant colonies were fixed with 4% paraformaldehyde and 90% ice-cold methanol, and then were stained with 1% crystal violet. Colonies with more than 50 cells were counted. Plating efficiency was defined as the percentage of cells that formed visible colonies. The surviving fraction of cells was determined by dividing the plating efficiency for each treatment by that of the unirradiated control. Ascorbic acid, a well-known antioxidant, was used as a positive control. The 25 μM concentration of ascorbic acid was used based on a previous report.[14]

2.4. Cell viability assay

LA-4 cells were seeded at a density of 3000 cells/well in 24-well plates. After 24 h, suplatast tosilate was added to the medium at final concentrations of 0, 10, 25, and 50 μg/mL for 2 h. Thereafter, cells were further exposed to a single dose of 30 Gy of irradiation, as described in the previous section. Cell viability was evaluated 72 h after irradiation using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), which identifies viable cells via a colorimetric method based on water-soluble tetrazolium salts.

2.5. Detection of intracellular ROS and DNA double-strand breaks

LA-4 cells were seeded at a density of 3000 cells/well in 8-well chamber slides. After 24 h, suplatast tosilate was added to the medium at final concentrations of 0 and 50 μg/mL, and the cells were exposed to a single dose of 30 Gy of irradiation. Intracellular ROS and DNA double-strand breaks (DSBs) were detected 48 h after irradiation using the ROS-ID® Oxidative Stress Detection Kit (Enzo Life Sciences, Farmingdale, NY) and OxiSelect DNA Double-Strand Break Staining Kit (Cell Biolabs, Inc., San Diego, CA) respectively in conjunction with fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan). Briefly, the former kit allows the detection of various types of intracellular ROS in the form of green fluorescence signals when cells are incubated with cell-permeable ROS detection reagents. The latter kit enables the detection of phosphorylated H2AX (γ-H2AX) as green fluorescence signals. H2AX, a histone H2A variant, is rapidly phosphorylated at sites of DNA damage when DSBs, which are probably the most pathogenic of the many different types of DNA damage that can occur within a cell, are introduced.[15] The fluorescence intensity and number of cells in the relevant area were then determined using an image analysis system (BZ-H2A/BZ-H1M/BZ-H1C, Keyence) attached to the fluorescence microscope. The fluorescence density, determined by dividing the total fluorescence intensity by the number of cells, was used to evaluate the fluorescence level in each group. Ascorbic acid (25 μM) was used as a positive control in these experiments.

2.6. Subcutaneous tumor model and treatments

The C57BL/6 mice were subcutaneously inoculated with 6.6×10^5 Lewis lung carcinoma cells in the lateral thorax. When the tumor volume reached approximately 60 mm^3, the mice were randomly assigned to the following three groups: (i) control group, group without treatment; (ii) RT group, group treated with RT alone; and (iii) RT + ST group, group treated with RT and continuous administration of suplatast tosilate since 3 days before RT. RT was performed for 5 consecutive days at a daily dose of 3 Gy with a lead shield to screen all areas except for the thoracic region where the tumor was located. The irradiation conditions were as detailed in the "Cell irradiation and clonogenic assay" section, except that the dose rate was 1.4–1.6 Gy/min. Based on the appropriate calculations, suplatast tosilate was dissolved in drinking water to administer a dosage of approximately 100 mg/kg/day, and the mice were given *ad libitum* access to the water throughout the experiment. The tumor size was measured every 2 days by using calipers, and the tumor volume, V, was calculated using the following formula: $V = \text{length} \times \text{width} \times \text{height} \times 0.5$.

2.7. Radiation-induced lung injury model

The C57BL/6 mice were randomly assigned to the three groups described above: control group, RT group, and RT + ST group. Radiation was delivered at a single dose of 15 Gy to the whole thorax. The irradiation conditions and the drug administration method were as described in the

“Subcutaneous tumor model and treatments” section. At 0, 6, 12, 18, 24, and 30 weeks after irradiation, the mice were euthanized and the harvested lungs were stored frozen at -80 °C until further measurements.

2.8. Bronchoalveolar lavage and cell analysis

The trachea was exposed and cannulated with an 18-gauge needle. The lungs were lavaged three times with 0.5 mL of phosphate-buffered saline (PBS). Bronchoalveolar lavage fluid (BALF) samples were pooled and centrifuged at 300 ×*g* for 5 min at 4 °C. The supernatants were stored at -80 °C for the measurement of cytokine concentrations. The cell pellets were resuspended in 1 mL of DMEM, and the total cell numbers were determined with a hemocytometer. Differential cell counts were performed by counting at least 300 cells on a smear prepared using cytospin (Thermo Fisher Scientific, Waltham, MA) and stained with Diff-Quick (Kokusai Shiyaku, Kobe, Japan).

2.9. Measurement of superoxide dismutase activity

The right lung of the mice was homogenized on ice supplemented with 200 μL of sucrose buffer. After centrifugation at 10,000 ×*g* for 60 min, the supernatant of the homogenate was collected for analysis. Superoxide dismutase (SOD) activity was measured using the SOD Assay Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. In addition, the total protein concentration in the sample was measured using the BCA protein assay kit (Pierce, Rockford, IL). SOD activity was expressed as U/mg protein.

2.10. Enzyme-linked immunosorbent assay

Using the right lung homogenate, 8-isoprostane content was analyzed with the Oxiselect 8-iso-prostaglandin F2α ELISA Kit (Cell Biolabs, Inc.) according to the manufacturer’s protocol, and expressed as U/mg protein. Commercially available ELISA kits for IL-6, TGFβ, and MCP-1 (R&D Systems, Minneapolis, MN) were used to measure cytokine concentrations in BALF according to the manufacturer’s protocols.

2.11. Hydroxyproline assay

For a biochemical analysis of lung fibrosis, right lung tissues were evaluated for hydroxyproline content as described previously.[16] The results were expressed as micrograms of hydroxyproline per milligram of lung tissue.

2.12. Histological examination

The lung tissue specimens of the mice were fixed by inflation with buffered 10% formalin solution. After being embedded in paraffin, the sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome.

2.13. Statistical analysis

Statistical analyses were performed using JMP Pro 12 (SAS Institute Inc., Cary, NC). Data are presented as means ± SEM. Student's *t*-test or Mann–Whitney *U* test was used to evaluate statistical differences between the groups. The Kaplan–Meier method was used for the survival analysis and comparisons were made using a log-rank test. Differences were considered significant when the *p*-value was less than 0.05.

3. Results

3.1. Effects of suplatast tosilate on radiation-induced injury in murine alveolar epithelial cells

To verify the cytoprotective effects of suplatast tosilate on radiation-induced injury, the murine alveolar epithelial cells were exposed to irradiation with or without a medium containing suplatast tosilate. As shown in Figure 1A, the irradiated cells showed decreased cell proliferation, whereas treatment with suplatast tosilate or ascorbic acid recovered the decreased cell proliferation by almost same magnitude. Suplatast tosilate also recovered the radiation-induced reduction in cell viability in a dose-dependent manner (Figure 1B). To ascertain whether the cytoprotective effect of suplatast tosilate resulted from the suppression of oxidative stress and DNA damage, intracellular ROS and DSBs were evaluated by fluorescence microscopy at 48 h after irradiation. As shown in Figures 1C–1F, intracellular green fluorescence reduced in the cells treated with suplatast tosilate or ascorbic acid, indicating that suplatast tosilate could suppress intracellular ROS and DNA damage generated through radiation-induced injury.

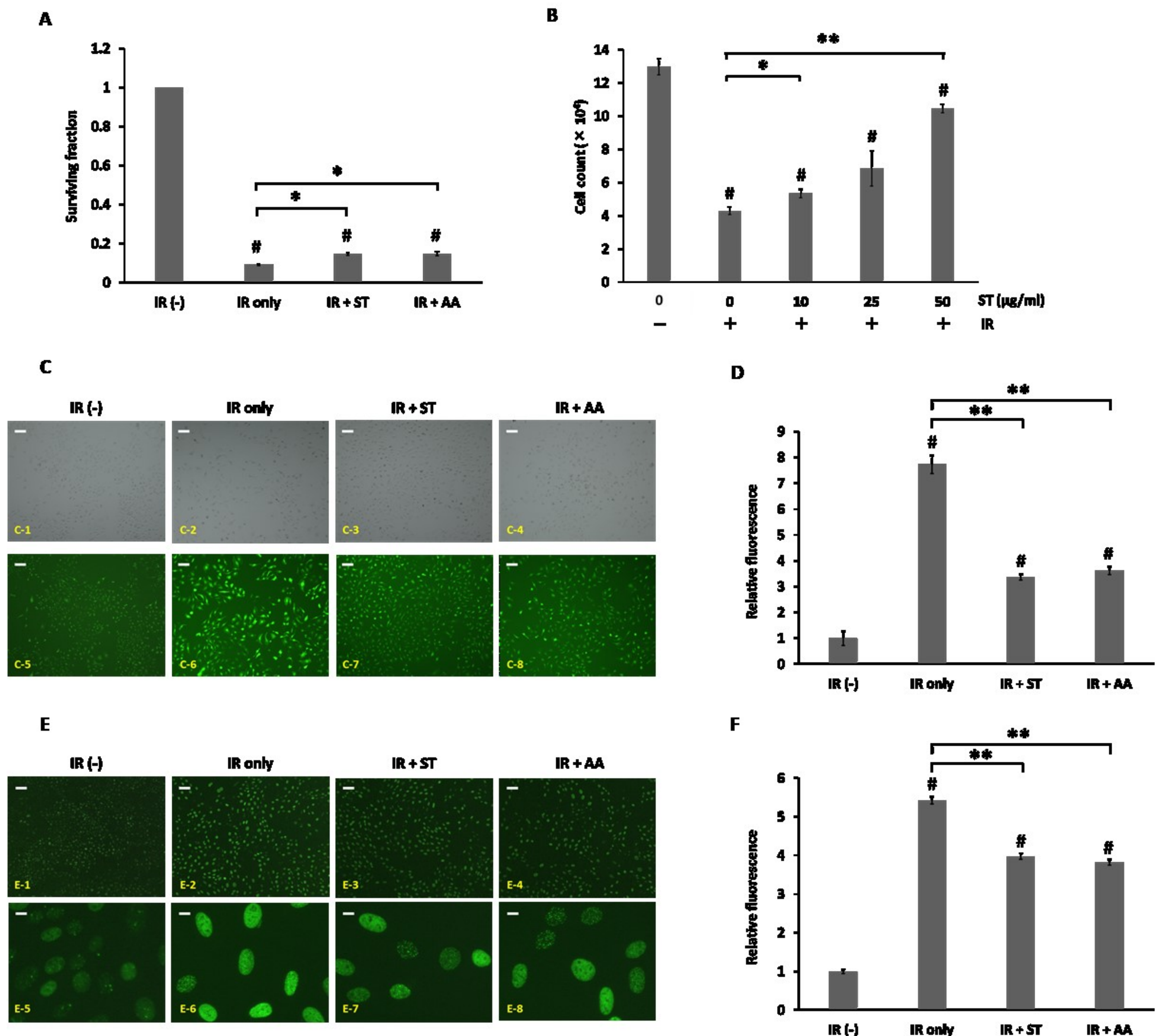

Figure 1. Effects of suplatast tosilate on radiation-induced injury in murine alveolar epithelial cells

(A) LA-4 cells were seeded at a density of 5000 cells/well in 24-well plates. After suplatast tosilate or ascorbic acid was added to the medium, the cells were exposed to a single dose of 5 Gy of irradiation. After 24 h, the cells were seeded in new 6-well plates and then allowed to grow for 10 days. The resultant colonies were stained and counted. Data are shown as means ± SEM (n = 4/group). (B) LA-4 cells were seeded at a density of 3000 cells/well in 24-well plates. After each concentration of suplatast tosilate was added to the medium, the cells were exposed to a single dose of 30 Gy of irradiation. Effects on cell viability were determined at 72 h after irradiation. Data are shown as means ± SEM (n = 6/group). (C) Intracellular ROS were evaluated by fluorescence microscopy at 48 h after irradiation. Green fluorescence indicates intracellular ROS. Representative

phase contrast images (C-1~4) and fluorescence images (C-5~8) are shown. Scale bars, 100 µm. (D) The fluorescence density in each group was quantified as described in Materials and Methods (n = 6/group). (E) Intracellular DSBs were evaluated by fluorescence microscopy at 48 h after irradiation. Green fluorescence indicates intracellular DSBs. Representative fluorescence images are shown. Scale bars are 100 µm for E-1~4 and 10 µm for E-5~8. (F) The fluorescence density in each group was quantified as described in the Materials and Methods (n = 4/group). *, $p < 0.05$, **, $p < 0.01$ between indicated groups; #, $p < 0.05$ versus no treatment group. IR, irradiation; ST, suplatast tosilate; AA, ascorbic acid; ROS, reactive oxygen species; DSB, DNA double-strand break.

3.2. Effects of suplatast tosilate on radiation sensitivity in the subcutaneous tumor model

To investigate if suplatast tosilate might diminish the efficacy of RT because of its antioxidant effects, the C57BL/6 mice were subcutaneously inoculated with Lewis lung carcinoma cells in the lateral thorax followed by irradiation and monitoring of the tumor volume. In the control group, the tumors markedly increased in size, whereas the tumor growth was suppressed in both RT and RT + ST groups at the same degree (Figure 2). All mice in the control group were euthanized at day 21 because of tumor growth. These findings indicated that suplatast tosilate did not affect the radiation sensitivity of the tumors.

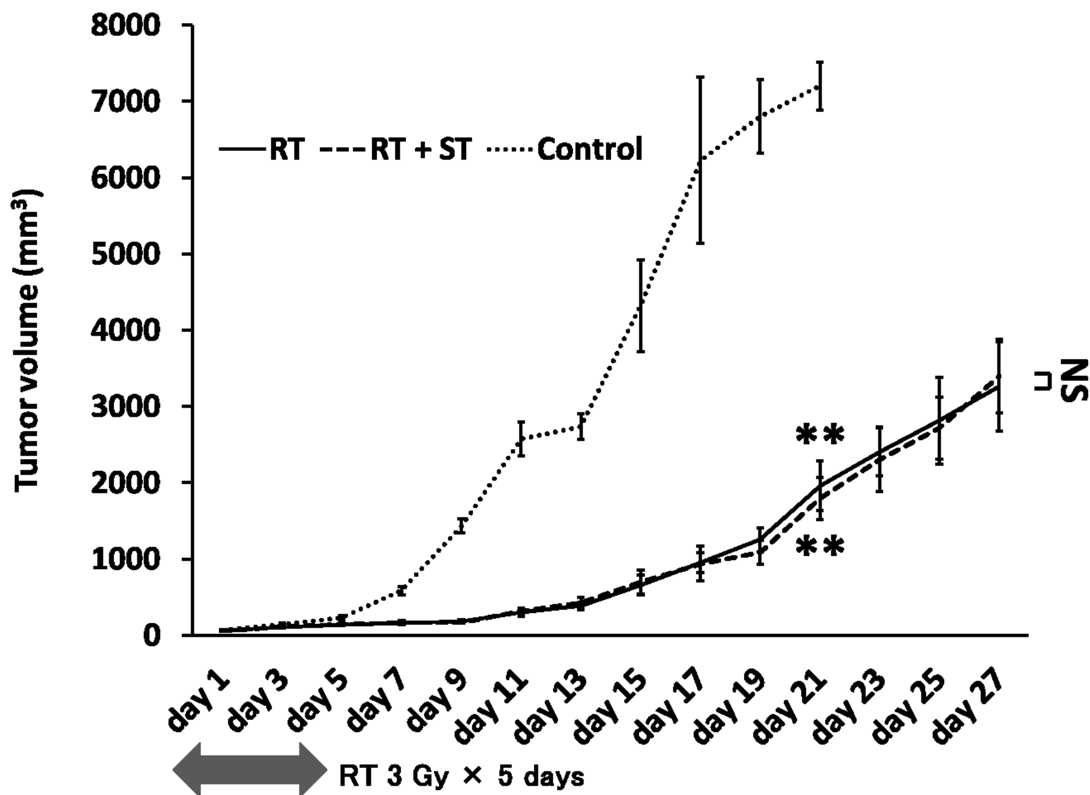

Figure 2. Effects of suplatast tosilate on radiation sensitivity in the subcutaneous tumor model
C57BL/6 mice were subcutaneously inoculated with 6.6×10^5 Lewis lung carcinoma cells in the lateral thorax. When the tumor volume reached approximately 60 mm^3, the mice were randomly assigned to the three groups as described in the Materials and Methods. Irradiation was performed for 5 days at a daily dose of 3 Gy. The tumor size was measured every 2 days. Data are shown as means ± SEM (n = 10 mice/group). **, $p < 0.01$ versus the control group at day 21. NS, not significant; RT, radiotherapy; ST, suplatast tosilate.

3.3. Effects of suplatast tosilate on survival rate in the mouse model of radiation-induced lung injury

To determine whether suplatast tosilate affected survival in the mouse model of radiation-induced lung injury, the survival rate was compared between irradiated mice supplemented with or without suplatast tosilate. The mice were administered a single irradiation of 15 Gy to the whole thorax and observed for 30 weeks. As shown in Figure 3, the survival rate in the RT + ST group was significantly higher than that in the RT group, which suggested that suplatast tosilate exerted protective effects against radiation-induced lung injury.

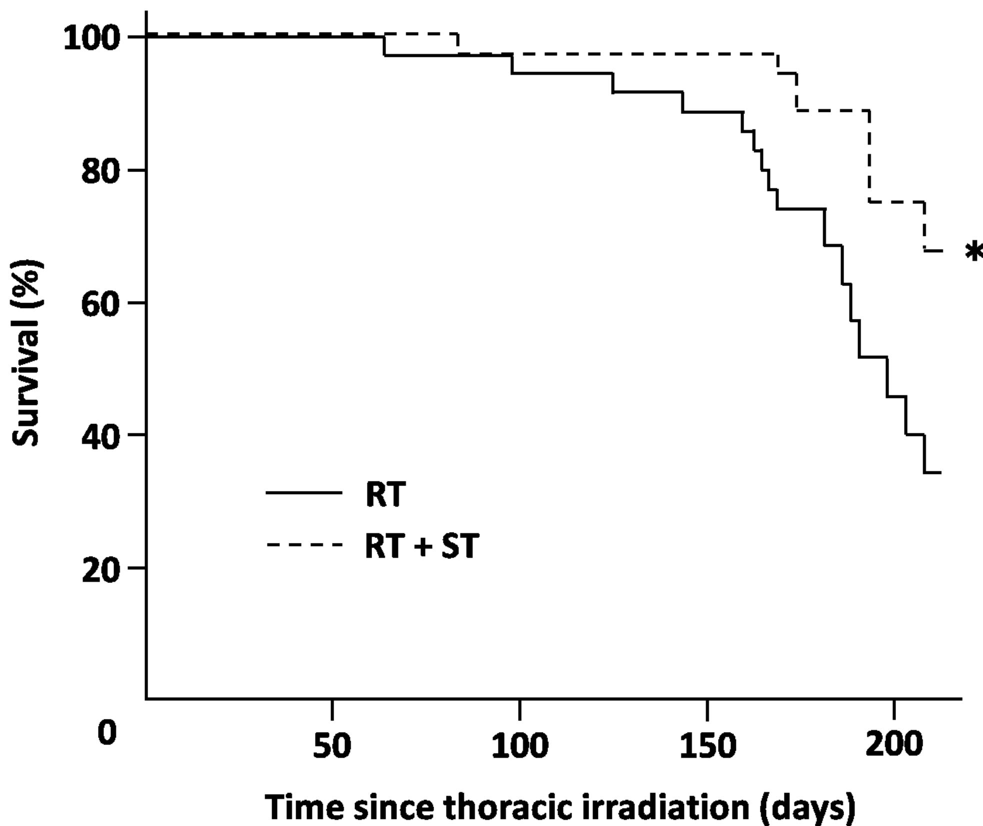

Figure 3. Survival analysis of mice after thoracic irradiation.
Survival was assessed in the RT and RT + ST groups after exposure to a single thoracic irradiation of 15 Gy (n = 35 mice/group). *, $p < 0.05$ versus the RT group. RT, radiotherapy; ST, suplatast tosilate.

3.4. Effects of suplatast tosilate on radiation-induced pulmonary inflammation

To evaluate the effects of suplatast tosilate on radiation-induced pulmonary inflammation, bronchoalveolar lavage (BAL) was performed before irradiation and 6, 12, 18, and 24 weeks after irradiation, after which cells in the BALF were analyzed. No major changes were found up to 12 weeks after irradiation. At 18 weeks after irradiation, the number of inflammatory cells (macrophages, lymphocytes, and neutrophils) in the BALF markedly increased in the RT group but significantly decreased in the RT + ST group (Figure 4).

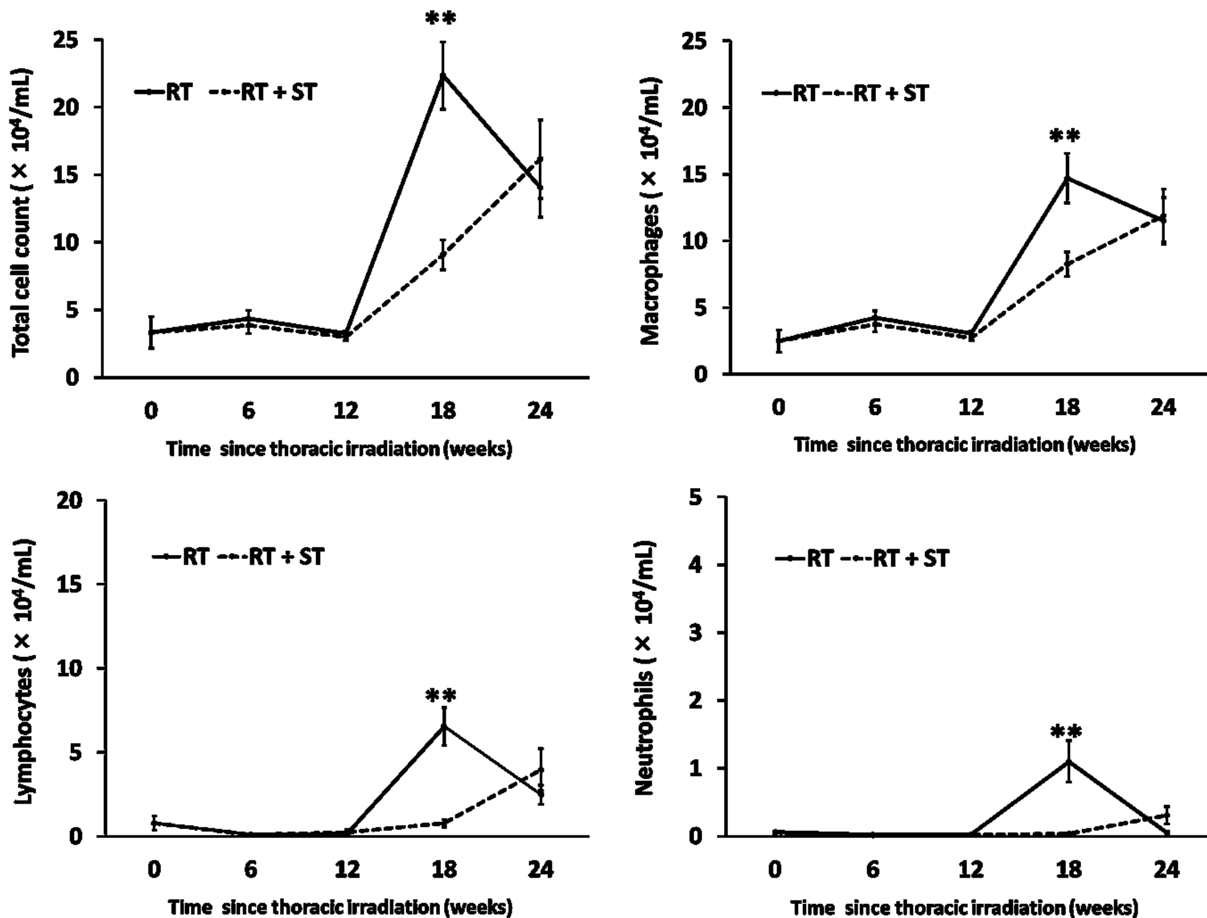

Figure 4. Effects of suplatast tosilate on inflammatory cells in bronchoalveolar lavage fluid after irradiation

Mice were exposed to a single thoracic irradiation of 15 Gy and administered water alone or water supplemented with suplatast tosilate (100 mg/kg/day). BALF samples were obtained before irradiation and 6, 12, 18, and 24 weeks after irradiation. Data are shown as means ± SEM (n = 5 mice/group at 0 and 24 weeks, n = 10 mice/group at the other time points). **, $p < 0.01$ versus the RT + ST group. RT, radiotherapy; ST, suplatast tosilate; BALF, bronchoalveolar lavage fluid.

3.5. Effects of suplatast tosilate on radiation-induced pulmonary fibrosis

To verify whether suplatast tosilate reduced radiation-induced pulmonary fibrosis, the mice were sacrificed 30 weeks after irradiation and hydroxyproline content was measured in the right lung. The hydroxyproline content in the RT group significantly increased compared to that in the control group. Thus, the development of radiation-induced pulmonary fibrosis in the RT group was confirmed (Figure 5A). In the RT + ST group, the increase in the hydroxyproline content was significantly suppressed compared to that in the RT group. A histological assessment further confirmed that the fibrotic area markedly reduced in the RT + ST group compared to that in the RT group, which was consistent with the hydroxyproline levels observed (Figure 5B).

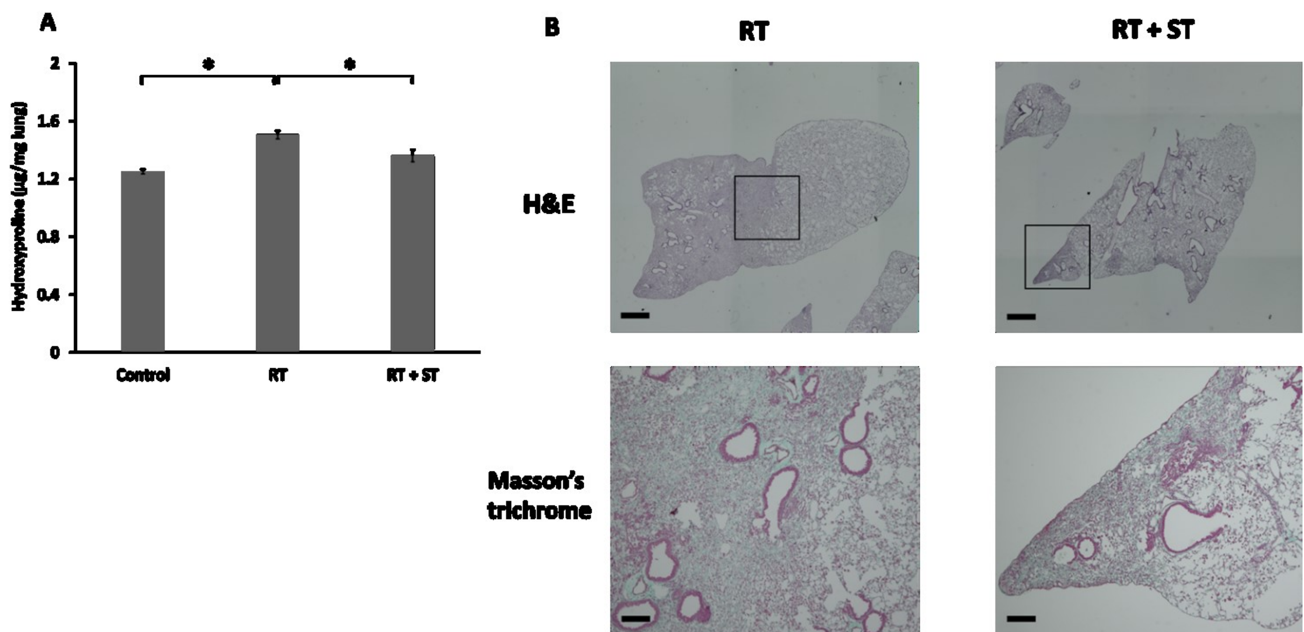

Figure 5. Effects of suplatast tosilate on radiation-induced pulmonary fibrosis

Mice were exposed to a single thoracic irradiation of 15 Gy and administered water alone or water supplemented with suplatast tosilate (100 mg/kg/day). (A) The mice were sacrificed 30 weeks after irradiation and hydroxyproline content in the right lung was measured. Data are shown as means ± SEM (n = 6 mice/group). *, $p < 0.05$. (B) The sections of the lung were stained with H&E and Masson's trichrome. One representative example of four or five is shown for the RT and RT + ST groups. Scale bars are 1000 μm for H&E and 200 μm for Masson's trichrome staining. RT, radiotherapy; ST, suplatast tosilate; H&E, hematoxylin and eosin.

3.6. Effects of suplatast tosilate on cytokine levels in the BALF after irradiation

Administration of suplatast tosilate markedly suppressed the number of inflammatory cells in the BALF at 18 weeks after irradiation. Consequently, the cytokine levels in the BALF were determined at this time point. The BALF levels of TGFβ, MCP-1, and IL-6 in the RT + ST group were significantly lower than those in the RT group (Figure 6).

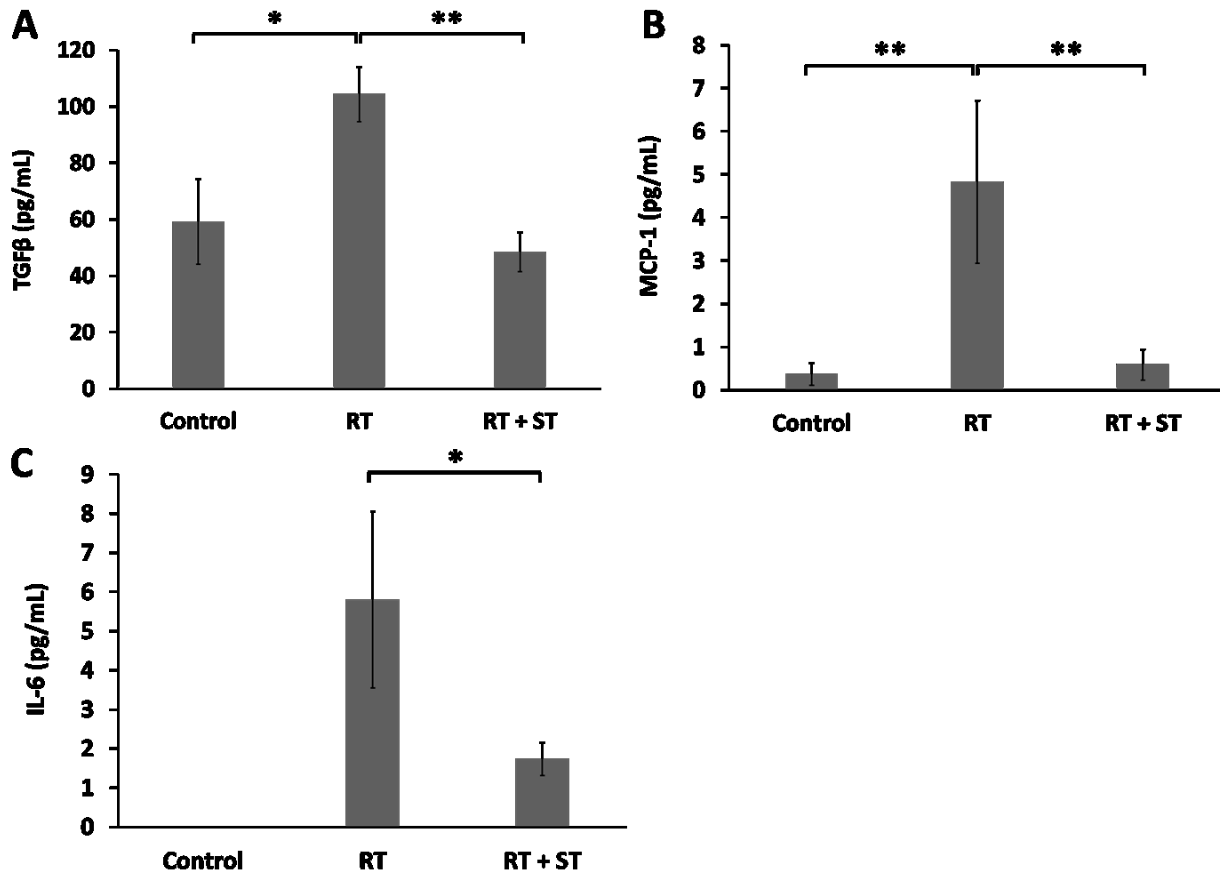

Figure 6. Effects of suplatast tosilate on cytokine levels in bronchoalveolar lavage fluid after irradiation

Mice were exposed to a single thoracic irradiation of 15 Gy and administered water alone or water supplemented with suplatast tosilate (100 mg/kg/day). BALF samples were obtained 18 weeks after irradiation. The concentrations of TGFβ (A), MCP-1 (B), and IL-6 (C) in the BALF were determined using commercially available ELISA kits. Data are shown as means ± SEM (n = 8 mice/group). *, $p < 0.05$; **, $p < 0.01$. RT, radiotherapy; ST, suplatast tosilate; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay.

3.7. Effects of suplatast tosilate on radiation-induced oxidative stress

At 18 weeks after irradiation, administration of suplatast tosilate suppressed both inflammatory cells and cytokines in the BALF, suggesting that the lung-protective mechanisms of suplatast tosilate might be attributable to suppression of oxidative stress by the agent at an earlier stage. To evaluate oxidative stress in the lungs, the level of 8-isoprostane, a marker of lipid oxidation, and the activity of SOD, an antioxidant enzyme that provides cellular defense against ROS, were measured in lung homogenates collected 6 weeks after irradiation. An increase in 8-isoprostane level and a decrease in SOD activity were observed in the RT group but the RT + ST group showed significant suppression of these parameters (Figure 7).

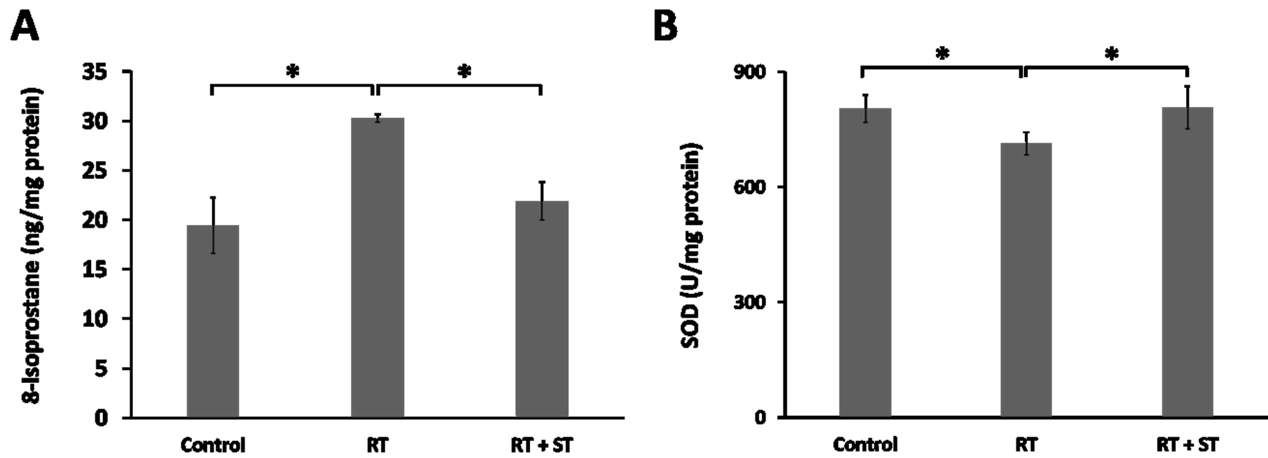

Figure 7. Effects of suplatast tosilate on radiation-induced oxidative stress

Mice were exposed to a single thoracic irradiation of 15 Gy and administered water alone or water supplemented with suplatast tosilate (100 mg/kg/day). The lung specimens were obtained 6 weeks after irradiation. The level of 8-isoprostane (A) and the activity of SOD (B) were measured in lung homogenates using commercially available kits. Data are shown as means ± SEM (n = 5 mice/group). *, $p < 0.05$. RT, radiotherapy; ST, suplatast tosilate; SOD, superoxide dismutase.

4. Discussion

In this study, treatment with suplatast tosilate recovered the decreased murine alveolar epithelial cell proliferation caused by irradiation via suppression of oxidative stress and DNA damage *in vitro*. *In vivo* experiments also demonstrated that suplatast tosilate reduced oxidative stress after thoracic irradiation without diminishing the antitumor efficacy of radiation, thereby suppressing inflammatory cells and cytokines in the BALF, preventing fibrosis, and improving the survival rate.

In addition to its direct harmful effects on DNA, radiation indirectly induces cell death by interacting with water molecules in tissues, which leads to the generation of ROS including hydrogen peroxide, superoxides, and hydroxyl radicals.[4,5] The harmful effects of ROS in living tissues are usually neutralized by endogenous antioxidants, which maintains oxidative homeostasis. However, when endogenous antioxidants are overwhelmed with excessive ROS, a state of oxidative stress develops.[17] The oxidative stress damage associated with excessive ROS is considered an important mechanism in radiation-induced lung injury.[18] The half-life of irradiation-generated ROS is short; however, it has been shown that irradiation-generated ROS trigger intracellular ROS production, presumably by the mitochondria, over several days.[19,20] In the present study, the irradiation-induced decrease in murine alveolar epithelial cell proliferation was significantly suppressed by suplatast tosilate. This cytoprotective effect of suplatast tosilate was accompanied by the suppression of intracellular ROS and DSBs levels after irradiation. Thus, the underlying mechanism of the improvement in cell proliferation was considered attributable to the suppression of irradiation-generated intracellular ROS and DNA damage induced by ROS, which is consistent with the ROS-scavenging activities of suplatast tosilate reported by Fukuhara et al.[11]

Although the underlying mechanism of progression of transient ROS generation after irradiation to chronic continuous oxidative stress has not been fully elucidated, one suggested causal factor is tissue hypoxia in connection with interstitial edema and decreased blood flow caused by the release of vasoconstrictive substances. In hypoxic tissue, accumulated and activated macrophages contribute to further ROS generation.[21–23] It is difficult to detect ROS themselves because of their short half-lives. Instead, oxides, produced when ROS react with DNA, lipids, and proteins, can be detected as indicators of ROS *in vivo*. Among such oxides, 8-isoprostane, produced by the ROS-mediated peroxidation of arachidonic acid in the phospholipids of the cell membrane, reflects accurate oxidative stress within tissues.[24] SOD, one of the most important antioxidant enzymes, catalyzes the dismutation of superoxide anions to form hydrogen peroxide and water.[25,26] Indeed in the current study, increased 8-isoprostane levels and decreased SOD activity in pulmonary tissues were observed at 6 weeks after irradiation. Suplatast tosilate significantly suppressed these changes induced by irradiation, suggesting that, even *in vivo*, suplatast tosilate suppressed radiation-induced oxidative stress.

ROS generated in association with irradiation induce inflammatory cytokines, growth factors, and fibrogenic cytokines[5,27]. It is suggested that not only ROS, but also DNA damage can induce immune responses, which result in the production of factors such as proinflammatory cytokines.[28,29] Among these cytokines, TGFβ plays a major role in the process of fibrosis. TGFβ is produced by various types of inflammatory, mesenchymal, and epithelial cells, and converts fibroblasts into myofibroblasts that produce the excessive extracellular matrix, which results in the induction of fibrosis.[30,31] MCP-1 is also produced by various types of cells and was initially identified as a monocyte chemotactic factor.[32] The MCP-1 level was reported to be elevated in BALF obtained from patients with idiopathic pulmonary fibrosis and MCP-1 stimulated collagen expression in lung fibroblasts.[33,34] Thus, MCP-1 is also involved in the pathogenesis of pulmonary fibrosis. Additionally, MCP-1 has been reported to be associated with radiation-induced lung toxicity in humans.[35] IL-6 is an acute phase proinflammatory cytokine produced by activated pulmonary macrophages, helper T-cells, pulmonary fibroblasts, and type-2 alveolar epithelial cells.[36,37] It is considered to be not only one of the most important factors of lymphocytic alveolar inflammation in lung injury but also involved in fibrosis in patients with idiopathic pulmonary fibrosis via the inhibition of the apoptosis of activated fibroblasts.[38,39] At 18 weeks after irradiation, suplatast tosilate significantly suppressed the increases in both numbers of inflammatory cells and levels of inflammatory and fibrogenic cytokines in BALF. In addition, at 30 weeks after irradiation, it was demonstrated that suplatast tosilate significantly suppressed the fibrosis in the lungs, which was confirmed by the suppression of the increase in hydroxyproline content and by a histological assessment. Collectively, our results indicated that suplatast tosilate reduced pulmonary oxidative stress in mice after irradiation and therefore suppressed inflammation and fibrosis.

This study has the following limitations. (i) The administered dose of suplatast tosilate (100 mg/kg/day) was markedly higher than the clinical dose (300 mg/day, approximately 6 mg/kg/day). Although the same dose was used in previous studies and the dose showed the highest efficacy for the treatment of asthma in a murine model, a treatment period was less than 3 weeks in these previous studies.[10,11,40] The extended treatment period of 30 weeks in this study did not give rise to any obvious problems, which increases the likelihood of the safe clinical application of suplatast tosilate. However, further investigation is needed to ascertain whether the suppression of radiation-induced lung injury can be achieved with lower dosages. (ii) Suplatast tosilate was dissolved in drinking water and then provided to the mice *ad libitum*. Therefore, although the calculated dose was 100 mg/kg/day, it is probable that the actual intake varied between individual mice. (iii) A single thoracic irradiation of 15 Gy to mice is known to result in the death of 30% to 40% of the mice by 24 weeks after irradiation, and the results of the present study were consistent with the notion.[41] We chose the radiation dose of 15 Gy based on both this notion and the results of our previous study.[42] Although this dose is considered appropriate for model establishment and judgment of efficacy of suplatast tosilate, most lung cancers and other types of malignant thoracic

tumors are usually treated using fractionated RT. It is unknown whether suplatast tosilate can suppress radiation-induced lung injury when fractionated irradiation, which is more similar to the clinical application regimen, is used. (iv) Since suplatast tosilate suppressed radiation-induced DSBs in irradiated murine alveolar epithelial cells, it is possible that the anti-inflammatory effects of suplatast tosilate in mice are attributable to the suppression of DNA damage, although testing this possibility merits further verification studies *in vivo*. (v) Oxidative stress markers in lung tissues and cytokine levels in BALF were only measured at a single time point; it is probable that a serial evaluation of these markers would enable a more detailed evaluation of pathology.

5. Conclusions

This study showed that administration of suplatast tosilate reduced oxidative stress in radiation-induced lung injury and therefore consecutively suppressed inflammation and fibrosis in mice. Oxidative stress has been suggested to be involved in various diseases, such as idiopathic pulmonary fibrosis and arteriosclerosis.[27] The antifibrotic effects of suplatast tosilate that are mediated through its antioxidant effects might also be useful in the treatment of oxidative stress-mediated diseases. However, further research is needed on this topic.

Acknowledgments

We thank Yukari Iyanaga for excellent technical assistance.

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