Plasminogen activator inhibitor-1 serves an important role in radiation-induced pulmonary fibrosis

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Abstract. Radiation-induced pulmonary fibrosis is a serious complication. Plasminogen activator inhibitor-1 (PAI-1) has been indicated to be a key factor in the progression of pulmonary fibrosis. In the present study, the effect of PAI-1 deficiency on radiation-induced pulmonary fibrosis was analyzed. Wild-type (WT) and *PAI-1*-deficient (PAI-1$^{-/-}$) mice were treated with thoracic irradiation of 15 Gy to induce pulmonary fibrosis. Analyses of bronchoalveolar lavage (BAL) fluids were performed 0, 4, 12, 18, and 24 weeks after irradiation. The degree of pulmonary fibrosis was assessed according to the histology of lung tissues and hydroxyproline contents. The results demonstrated that the irradiation of WT mice increased PAI-1 expression in the lungs after 18 weeks and established lung fibrosis at 24 weeks. The number of total cells and transforming growth factor-β levels in BAL fluid were significantly lower at 24 weeks after irradiation in PAI-1$^{-/-}$ mice compared with WT mice. Furthermore, histological examination revealed that the extent of pulmonary fibrosis was attenuated in PAI-1$^{-/-}$ mice compared with that in WT mice. Hydroxyproline content was also significantly lower in PAI-1$^{-/-}$ mice compared with WT mice at 24 weeks after irradiation. In conclusion, PAI-1 serves an important role in the development of radiation-induced pulmonary fibrosis and may represent a novel therapeutic target for pulmonary fibrosis.

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Introduction

Radiation therapy is an important treatment modality for thoracic cancers. However, radiation-induced pneumonia and subsequent pulmonary fibrosis can be serious and fatal complications, and they are major dose-limiting factors of radiotherapy. Radiation-induced pulmonary fibrosis, characterized by inflammatory cell infiltration, fibroblast proliferation, and excessive deposition of extracellular matrix (ECM) proteins such as collagen in the lung parenchyma (1-3), presents 6 to 24 months after irradiation and continues to progress over a period of years (4). The pathological mechanisms of radiation-induced pulmonary fibrosis are complex and involve numerous cell types. A large number of studies have presented evidence showing the involvement of several mediators in the pathogenesis of this disease (1,5). Nonetheless, there is not an established treatment protocol for radiation-induced pulmonary fibrosis. Therefore, the development of therapeutic strategies for this disease is urgently needed.

Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of the plasminogen activator system, which blocks fibrinolysis and promotes ECM accumulation in tissues (6-8). There is a growing body of evidence demonstrating that PAI-1 plays a crucial role in pulmonary fibrosis. Indeed, it has been shown that overexpression of *PAI-1* enhances bleomycin (BLM)-induced pulmonary fibrosis (9). Moreover, the inhibition of *PAI-1* expression via gene deletion (9,10), intrapulmonary administration of small interfering RNAs (11), or a specific PAI-1 inhibitor (12) attenuates the development of BLM-induced pulmonary fibrosis in mouse models. *In vitro*, PAI-1 is reported to contribute to the development of pulmonary fibrosis by promoting ECM accumulation and is associated with the epithelial-mesenchymal transition (EMT) of lung epithelial cells and differentiation of fibroblasts to myofibroblasts (7,12).

Several studies have demonstrated the involvement of PAI-1 in radiation-induced tissue injury and fibrosis (13,14). The overexpression of PAI-1 has been reported to lead to the development of radiation-induced nephrosclerosis (15,16) and enteritis (17). These observations suggest that PAI-1 plays an

important role in radiation-induced tissue injury. However, the association between PAI-1 and radiation-induced pulmonary fibrosis has not been fully elucidated. In this study, we investigated the effect of *PAI-1* gene deficiency on radiation-induced lung fibrosis by analyzing the degree of lung fibrosis induced by irradiation in *PAI-1* knockout and wild-type (WT) mice.

Materials and methods

Animal model of radiation-induced lung fibrosis. PAI-1-deficient (PAI-1$^{-/-}$) C57BL/6 mice were purchased from Jackson Laboratory, (Bar Harbor, ME, USA) and bred according to methods approved by the Institutional Animal Care and Use Committee of Hiroshima University (Hiroshima, Japan). All animal experiments were approved by the animal ethics committee of Hiroshima University (permit no: A13-98). Age-, sex-, and body weight-matched WT C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan). PAI-1$^{-/-}$ mice and WT mice were anesthetized with intraperitoneal injection of pentobarbital sodium (30 mg/kg). For euthanasia of mice, the dose of 100-120 mg/kg pentobarbital sodium was used. A single dose of 15 Gy irradiation was delivered to the thorax of each mouse with an X-ray irradiator (MBR-1520R-3; Hitachi, Ltd., Tokyo, Japan). Irradiation characteristics were as follows: Beam energy, 150 kV; X-ray dose rate, 1.3 Gy/min; source-surface distance, 50 cm; diameter of the radiation field, 25 cm; filter, 0.5 mm Cu and 0.1 mm Al.

Analysis of bronchoalveolar lavage (BAL) fluid. BAL fluids were collected 0, 4, 12, 18, and 24 weeks after irradiation as previously described (11). Briefly, mice were sacrificed with a lethal dose of pentobarbital, the tracheas were cannulated with an 18-gauge needle, and the lungs were lavaged three times with 0.5 ml of phosphate-buffered saline (PBS). Lavage fluids were pooled and were cleared of cells via centrifugation. Cells in BAL fluids were counted with a standard hemocytometer. Differential cell counts were obtained by Diff-Quik (Kokusai Shiyaku, Kobe, Japan) using cytospin preparations (Shandon, Pittsburgh, PA, USA).

Measurements of concentrations of PAI-1, TGF-β, and albumin in BAL fluid. PAI-1 and TGF-β levels in BAL fluids were measured at 0, 4, 12, 18, and 24 weeks after irradiation using ELISA kits for PAI-1 (Innovative Research, Novi, MI, USA) and TGF-β (R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions. Mouse albumin concentrations in BAL fluids were measured with an ELISA kit according to the manufacturers' instructions (Shibayagi, Gunma, Japan).

Hydroxyproline assay. To quantify lung collagen contents, the hydroxyproline contents of the left lungs were measured in each group at 24 weeks after irradiation as described previously (10).

Histology. After BAL and lung perfusion, mouse lungs were fixed by inflation with a buffered 4% formalin solution. Lung tissue specimens were embedded in paraffin and cut into 5-μm sections, which were stained with hematoxylin and eosin or Masson's trichrome.

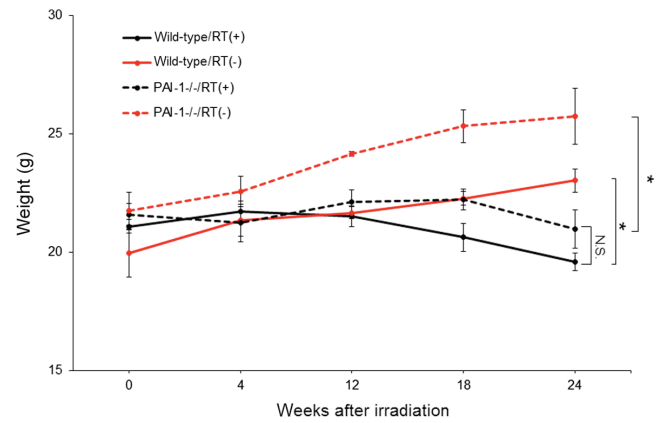

Figure 1. Changes in animal body weight after irradiation. Data are expressed as mean ± SEM. Comparison of the body weights at 24 weeks after irradiation was analyzed by Kruskal-Wallis test followed by Steel-Dwass test. *P<0.05. NS, not significant.

Statistical analysis. Results are expressed as means ± SEM. The Wilcoxon rank sum test was used to evaluate differences between two groups. Comparisons of the multiple groups were evaluated by using Kruskal-Wallis test followed by Steel-Dwass test or Steel test. Correlations were analyzed with Pearson's correlation coefficient test. The Kaplan-Meier method was used to analyze mouse survival curves. Differences in survival between two groups were analyzed using the log-rank test. All statistical analyses were performed using JMP Genomics v.7.0 software (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of thoracic irradiation on body weights of mice. The body weights of mice were measured over time to assess the effect of thoracic irradiation on body weight changes. Thoracic radiation reduced mouse body weights significantly in both WT and PAI-1$^{-/-}$ mice, with no difference observed between the two groups at 24 weeks after irradiation (Fig. 1).

Assessment of inflammatory cells in BAL fluid after thoracic irradiation. To assess the inflammatory response to thoracic irradiation, inflammatory cells in BAL fluids were analyzed at baseline and 4, 12, 18, and 24 weeks after irradiation. As shown in Fig. 2A, total cell counts in BAL fluids from PAI-1$^{-/-}$ mice peaked at 4 weeks after irradiation and subsequently declined. In contrast, total cell counts in BAL fluids from WT mice gradually increased to a peak at 18 weeks after irradiation. Total cell counts in BAL fluids were significantly higher at 4 and 12 weeks and significantly lower at 24 weeks after irradiation in PAI-1$^{-/-}$ mice than in WT mice. Based on differential cell counts in BAL fluids, numbers of macrophages were significantly higher at 4 and 12 weeks and significantly lower at 18 and 24 weeks after irradiation in PAI-1$^{-/-}$ mice than in WT mice, and numbers of lymphocytes were significantly higher at 4 and 12 weeks and significantly lower at 24 weeks after irradiation in PAI-1$^{-/-}$ mice than in WT mice.

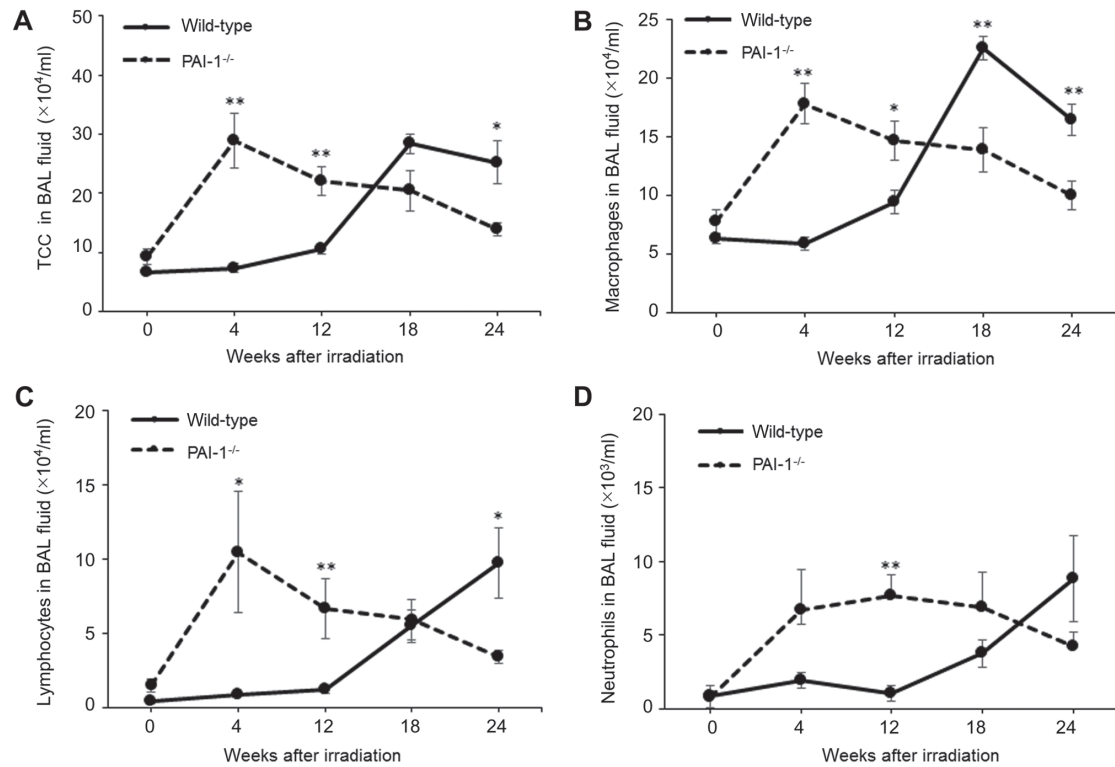*PAI-1 and TGF-β levels in BAL fluid.* To determine the effect of thoracic irradiation on the expression of PAI-1 and

Figure 2. Numbers of inflammatory cells in BAL fluids of WT and PAI-1-/- mice. (A) TCC, (B) macrophages, (C) lymphocytes and (D) neutrophils were measured in BAL fluid. Data are expressed as mean ± SEM. *P<0.05, **P<0.01. vs. WT at the same time point. WT, wild-type; PAI-1, plasminogen activator inhibitor-1; BAL, bronchoalveolar lavage; TCC, total cell counts.
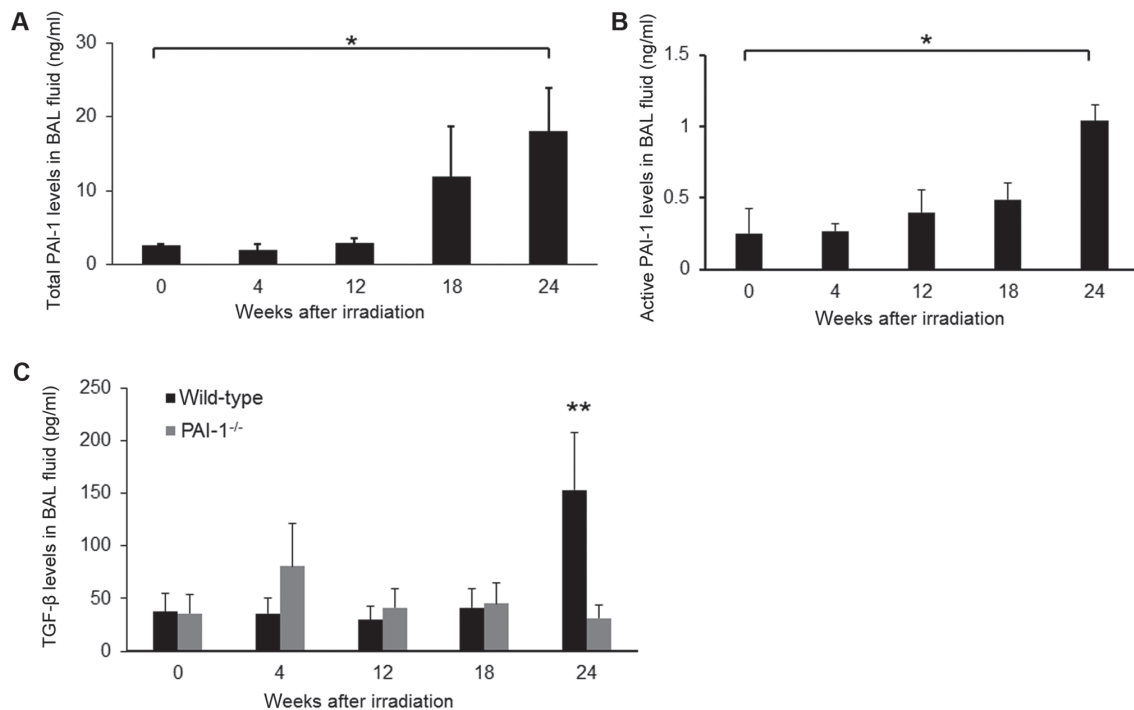

Figure 3. (A) Total PAI-1 and (B) active PAI-1 levels in BAL fluids of irradiated WT mice. Data are shown as mean ± SEM for 5-8 mice at 0, 4, 12, 18, and 24 weeks after irradiation. Comparisons of the various time points were analyzed by Kruskal-Wallis test followed by Steel test. *P<0.05 vs. the control (time 0). (C) TGF-β levels in BAL fluids of irradiated WT and PAI-1-/- mice. Data are shown as mean ± SEM for 5-8 mice per group at 0, 4, 12, 18, and 24 weeks after irradiation. **P<0.01 vs. PAI-1-/- at the same time point. WT, wild-type; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; BAL, bronchoalveolar lavage.

TGF-β in the lung, PAI-1 and TGF-β levels in BAL fluid were measured using ELISA kits. In WT mice, PAI-1 levels in BAL fluids were unchanged from baseline until 12 weeks after irradiation but started increasing after 18 weeks and
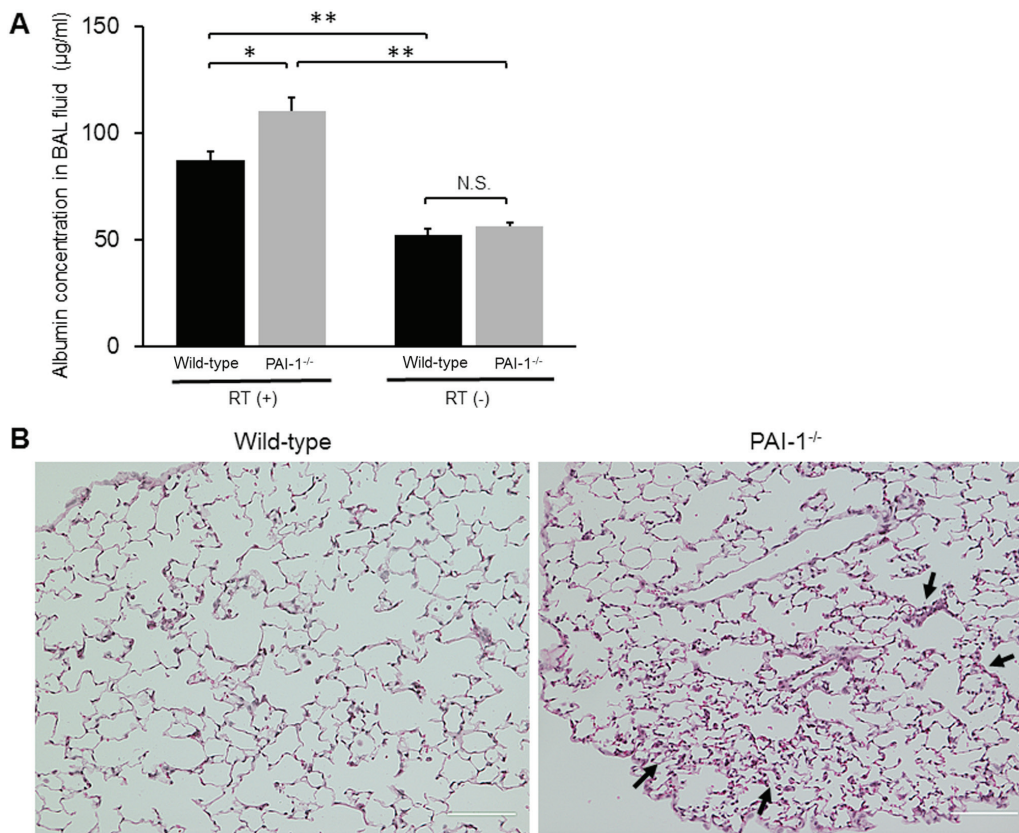
Figure 4. (A) Albumin concentration in BAL fluids of WT and PAI-1$^{-/-}$ mice at 4 weeks after irradiation and non-irradiated age-matched control mice. Data are shown as mean ± SEM for 4-6 mice per group. *P<0.05, **P<0.01 as indicated. (B) Histological analyses using hematoxylin and eosin staining in sections of irradiated lung at 4 weeks after irradiation. Magnification: x200. Scale bar=100 μm. Arrows show the areas of inflammatory cell infiltration. WT, wild-type; NS, not significant; PAI-1, plasminogen activator inhibitor-1; BAL, bronchoalveolar lavage.

reached their highest levels at 24 weeks after irradiation. PAI-1 levels at 24 weeks were significantly higher compared with those before irradiation (Fig. 3A). TGF-β levels in BAL fluids from WT mice showed the same trend (Fig. 3B). In contrast, TGF-β levels in BAL fluids from PAI-1$^{-/-}$ mice were almost unchanged until 24 weeks after irradiation, at which point they were significantly lower than those in WT mice (31.51±7.18 vs. 152.95±70.05 pg/ml; P=0.002; Fig. 3C).

Effects of PAI-1 deficiency on early-phase lung injury after irradiation. As mentioned above, we found that the number of inflammatory cells increased 4 weeks after irradiation. Therefore, to investigate the degree of inflammation in the lungs, we analyzed the concentration of albumin in BAL fluids and assessed histological changes in the lungs 4 weeks after irradiation. Albumin concentrations in BAL fluids were significantly higher in PAI-1$^{-/-}$ mice than in WT mice (110.62±5.98 vs. 87.34±4.31 μg/ml; P=0.01; Fig. 4A). In addition, histological examination of lungs showed that the degree of inflammatory cell infiltration into the alveoli was higher in PAI-1$^{-/-}$ mice than in WT mice (Fig. 4B).

Effect of PAI-1 deficiency on radiation-induced pulmonary fibrosis. To assess the degree of radiation-induced pulmonary fibrosis, right lungs were histologically examined, and the hydroxyproline contents of left lungs were measured at 24 weeks after irradiation. Representative histological images of lungs revealed that the extent of pulmonary fibrosis was

apparently attenuated in PAI-1$^{-/-}$ mice compared with that in WT mice (Fig. 5A). In addition, the hydroxyproline contents of the left lung per mg body weight were found to be significantly lower in PAI-1$^{-/-}$ mice than in WT mice at 24 weeks after irradiation (3.75±0.45 vs. 4.66±0.57 μg/g body weight; P=0.031; Fig. 5B). The hydroxyproline content was adjusted for mouse body weight as previously reported (18). Actually, we confirmed that there was a correlation between hydroxyproline content and body weight (r=0.665, P=0.036; Fig. 5C).

Effect of thoracic irradiation on mouse mortality. In PAI-1$^{-/-}$ mice, a high mortality rate was observed from 4 to 12 months after irradiation due to inflammation in the lungs. In contrast, in WT mice, increased mortality was observed 18 weeks after irradiation due to lung fibrosis. However, there was no significant difference in the survival rates of the two groups at 24 weeks after irradiation (P=0.322; Fig. 6).

Discussion

In the present study, we showed that pulmonary fibrosis developed at 24 weeks after thoracic irradiation in WT mice with accompanying elevations in both PAI-1 and TGF-β in BAL fluid; however, in PAI-1$^{-/-}$ mice, the degree of pulmonary fibrosis was limited, and there was no elevation in TGF-β in BAL fluid. These data indicate the direct involvement of PAI-1 in the development of radiation-induced pulmonary fibrosis.
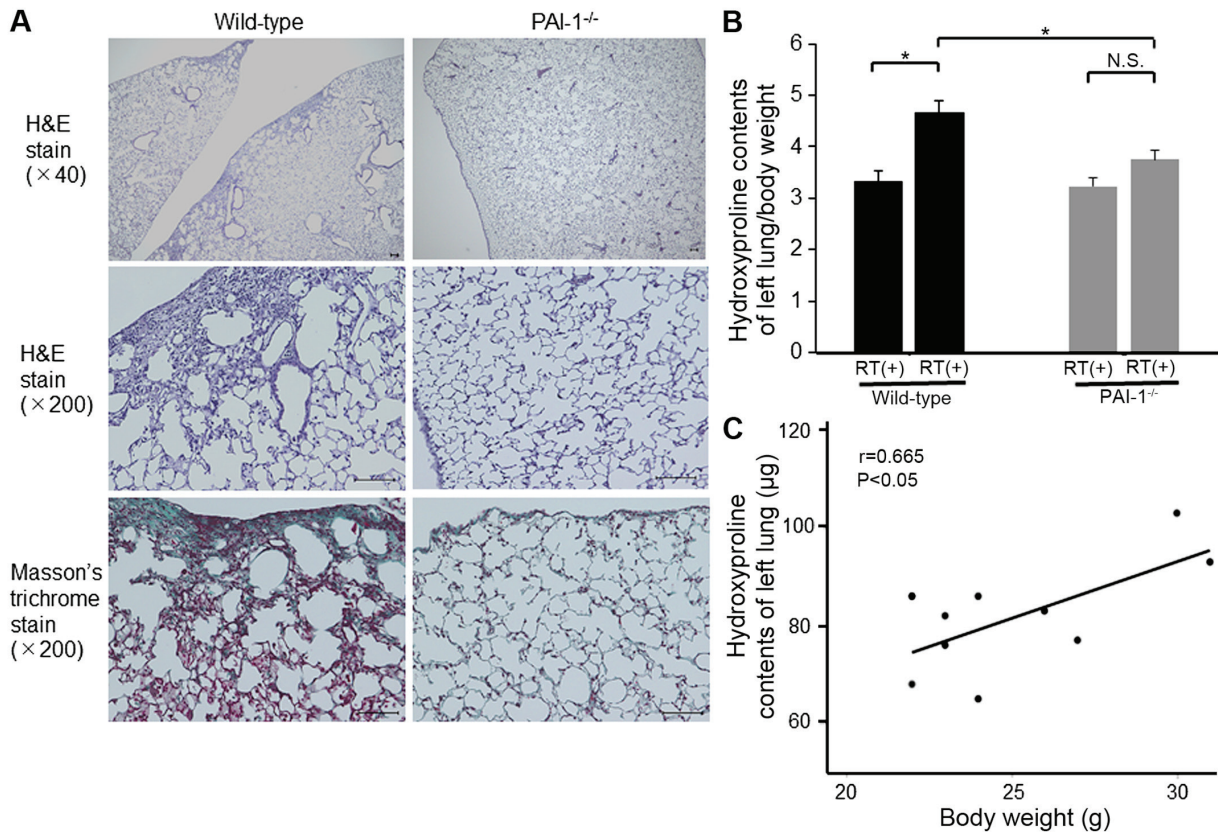
Figure 5. (A) Histological analyses using hematoxylin and eosin or Masson's trichrome staining in sections of irradiated lung at 24 weeks after irradiation. Scale bar=100 μm. (B) Hydroxyproline contents of the left lung per body weight at 24 weeks after irradiation. Data are shown as mean ± SEM for 5-6 mice per group. *P<0.05 as indicated. (C) Correlation between hydroxyproline contents of the left lung and body weight in mice (n=10). PAI-1, plasminogen activator inhibitor-1; H&E, hematoxylin and eosin.
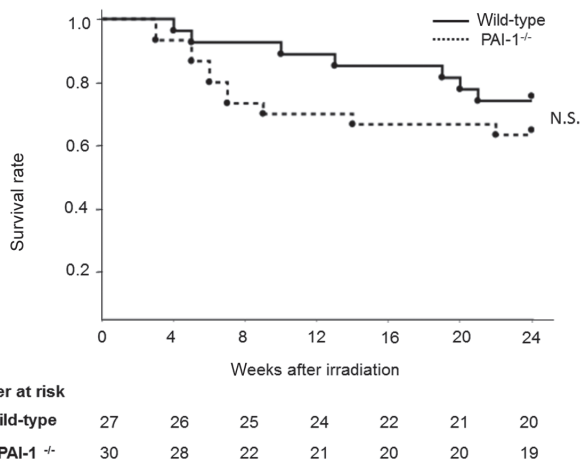

Figure 6. Kaplan-Meier survival curves of WT (n=27) and PAI-1⁻/⁻ (n=30) mice after thoracic irradiation. WT, wild-type; NS, not significant; PAI-1, plasminogen activator inhibitor-1.

We demonstrated that PAI-1 gene deficiency reduced the progression of radiation-induced fibrosis in this study. Previous studies showed that PAI-1 deletion or suppression using siRNA or a specific PAI-1 inhibitor limited the development of BLM-induced pulmonary fibrosis in murine models (9-11). In the field of radiation-induced pulmonary fibrosis, there are several studies that have investigated whether PAI-1 is associated with radiation-induced

pulmonary fibrosis. In animal models, the intra-abdominal administration of recombinant truncated PAI-1 protein or pentoxifylline, an antifibrotic agent, attenuates radiation-induced fibrosis in the lungs by reducing PAI-1 activity and promoting fibrin proteolysis (19,20). In contrast, genetic deficiency of nuclear factor erythroid 2-related factor 2 (NRF2) increases PAI-1 expression in irradiated murine lungs, resulting in the development of late tissue injury in mice (21). These results suggest that PAI-1 may be promising as a therapeutic target for radiation-induced pulmonary fibrosis. This is the first study to investigate the effect of PAI-1 deficiency on the progression of radiation-induced pulmonary fibrosis.

Several studies have shown that transforming growth factor beta (TGF-β) is the major cytokine responsible for pulmonary fibrosis after irradiation (1-3,22,23). Furthermore, TGF-β is known to be a potent inducer of PAI-1 expression in various diseases associated with fibrosis (7,24). A previous study showed that both TGF-β and PAI-1 expression levels were increased in the skin of irradiated rats (25). In addition, the administration of anti-TGF-β antibody limited radiation-induced PAI-1 expression and the degree of skin fibrosis in this study. These results indicate that PAI-1 is associated with the process of radiation-induced tissue fibrosis as a downstream effector of TGF-β.

In the present study, we demonstrated that the degree of pulmonary fibrosis and the levels of TGF-β in BAL fluid significantly increased in WT mice after irradiation. In

contrast, these results were not observed in PAI-1$^{-/-}$ mice. In addition, a previous report also showed that the inhibition of PAI-1 activity via systemic delivery of recombinant truncated PAI-1 protein attenuated pulmonary fibrosis and the expression of TGF-β in BAL fluids of irradiated mice (19). These results suggest that PAI-1 is not only a downstream effector of TGF-β but is also involved in radiation-induced pulmonary fibrosis through the regulation of TGF-β expression. In fact, in mesangial cells, PAI-1 is reported to activate the *TGF-β1* gene promoter (26) and enhance the expression of TGF-β (27). Furthermore, we previously showed that exogenous TGF-β induced EMT and stimulated the production of endogenous TGF-β in A549 cells. In contrast, a PAI-1-specific inhibitor limited TGF-β-induced EMT and the production of TGF-β in A549 cells (12). These observations imply that PAI-1 would be an effective treatment target for pulmonary fibrosis as a regulator of TGF-β.

In this study, the numbers of total cells, lymphocytes and macrophages, as well as the albumin levels in BAL fluids were significantly higher and the degree of infiltration of inflammatory cells was histologically more prominent at 4 weeks after irradiation in PAI-1$^{-/-}$ mice than in WT mice. Furthermore, several PAI-1$^{-/-}$ mice died between 4 and 12 months after irradiation due to inflammation in the lungs. These findings are compatible with those of a previous study that showed that the numbers of total cells, neutrophils, and macrophages in BAL fluids were significantly higher in PAI-1$^{-/-}$ mice than in WT mice with acid-induced acute lung injury (28). Although in these studies the extent of pulmonary fibrosis was lower in PAI-1$^{-/-}$ mice than in WT mice, these findings suggest that PAI-1 inhibition should be avoided in the early phase after irradiation. To elucidate the mechanism by which an acute inflammatory response is induced in lung injuries by *PAI-1* deficiency, further examination is needed.

Radiation-induced pulmonary fibrosis is a chronic progressive condition resulting from high levels of reactive oxygen species (ROS) induced by irradiation and the subsequent injury of alveolar epithelial cells and vascular endothelial cells (29). The underlying mechanism of radiation-induced pulmonary fibrosis is considered to be similar to that of idiopathic pulmonary fibrosis (IPF). In the present study, we examined the involvement of PAI-1 and TGF-β, which activate fibroblasts, induce EMT in epithelial cells, and accelerate the coagulation cascade, in the process of pulmonary fibrosis. However, a large number of other factors have been reported to be associated with pulmonary fibrosis. Previous studies have shown that several mediators, such as platelet-derived growth factor, vascular endothelial growth factor, and fibroblast growth factor, are also involved in fibroblast activation in IPF progression (30,31). Furthermore, it has been reported that endoplasmic reticulum stress in alveolar type II cells (32) and T lymphocytes, such as Th-2 and Th-17 T-cells, are also implicated in pulmonary fibrosis (31). Further studies are therefore required to elucidate the involvement of these factors in radiation-induced pulmonary fibrosis.

In conclusion, our study shows that *PAI-1* genetic deficiency attenuates the development of pulmonary fibrosis after irradiation. These results suggest that PAI-1 may represent a novel therapeutic target for radiation-induced pulmonary fibrosis.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SS and NH designed the present study. SS performed the experiments. SS, TM, TS, YH, SM, TN, HI, KF, HH and NH contributed to analysis and interpretation of the data. SS, TM, TS, YH, SM, TN, HI, KF, HH and NH drafted the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the animal ethics committee of Hiroshima University (permit no: A13-98) and animals were maintained according to guidelines for the Ethical Use of Animals in Research at Hiroshima University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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