Protective Effect of Urinary Trypsin Inhibitor on the Development of Radiation-Induced Lung Fibrosis in Mice

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Urinary trypsin inhibitor/Radiation induced lung fibrosis/Protector/Normal tissue effect.

This study aimed to analyze whether Ulinastatin, a urinary trypsin inhibitor (UTI), inhibits the TGF-β signaling pathway and lung fibrosis induced by thoracic irradiation in a lung injury mouse model. The throraces of 9-week-old female fibrosis-sensitive C57BL/6 mice were irradiated with a single X-ray dose of 12 Gy or 24 Gy. UTI was administrated intraperitoneally at a dose of 200,000 units/kg concurrently with radiation (concurrent UTI) or daily during the post-irradiation period for 8–14 days (post-RT UTI). Mice were sacrificed at 16 weeks after irradiation to assess the histological grade of lung fibrosis and immunohistochemical TGF-β expression. Survival rates of mice given 24 Gy to the whole lung ± UTI were also compared. Post-RT UTI reduced the score of lung fibrosis in mice, but concurrent UTI had no beneficial effects in irradiated mice. The fibrosis score in post-RT UTI mice was 3.2 ± 1.0, which was significantly smaller than that of irradiated mice without UTI treatment (RT alone; 6.0 ± 1.3; p < 0.01). The rates of TGF-β positive cells in post-RT UTI and the RT alone mice were 0.18 ± 0.03 and 0.23 ± 0.04, respectively (p < 0.01). There was a significantly positive correlation between the fibrosis score and the TGF-β positive rate (R² = 0.26, p < 0.01). The survival rate at 30 weeks for post-RT UTI mice was significantly better than that of RT alone mice (33% vs. 10%, p < 0.05). The administration of post-RT UTI suppressed TGF-β expression and radiation-induced lung fibrosis, which resulted in significant survival prolongation of the irradiated mice.

INTRODUCTION

Radiation-induced lung injury is one of the major dose-limiting factors of radiotherapy for thoracic malignancies such as lung and breast cancers.1,2) Radiation-induced lung fibrosis develops several months to years after radiation exposure at least in the irradiated field. Moreover, there are non-negligible critical risks of developing generalized lung fibrosis that is usually life-threatening.3) Although many studies have tried to analyze the mechanisms underlying the pathogenesis of radiation-induced lung fibrosis, the mechanisms still remain unclear. Hence, the prevention of radiation-induced lung fibrosis is difficult to realize in a clinical setting although extensive efforts have been undertaken in the exploration of this condition. Corticosteroids are the mainstay of treatment for radiation pneumonitis, and approximately 80% of patients respond to treatment. However, the use of corticosteroids as prophylaxis is not substantially, if any, benefit.4) Hence, the development of preventive and/or therapeutic strategies, including the development of new drugs, is an urgent matter in the field of radiation therapy for lung cancer.

Transforming growth factor beta (TGF-β) is an immunomodulatory cytokine regulating the proliferation and differentiation of various cell types. It also contributes to the maintenance of tissue architecture by influencing the production of extracellular matrix components. TGF-β is proposed to play an important role in radiation-induced lung fibrosis. Bronchial epithelial cells contained the largest amounts of TGF-β and alveolar macrophages are suggested as potential TGF-β-producing cells5,6) that may serve as a stimulus for the persistent expression of connective tissue genes (fibronectin, procollagen, and smooth muscle actin).

Ulinastatin, a glycoprotein with a molecular weight of...
67 kDa purified from human urine, is used for the treatment of acute pancreatitis and acute disturbances of systemic circulation.\textsuperscript{7} Ulinastatin referred to as urinary trypsin inhibitor (UTI) was also reported to decrease the production of cytokines, such as tumor necrosis factor alpha, interleukin-1 alpha, and interleukin-1 beta.\textsuperscript{8,9} UTI has been widely used as a drug for patients with acute inflammatory disorders such as disseminated intravascular coagulation, shock, and pancreatitis.\textsuperscript{10} Protease inhibitor or UTI decreased the inflammatory reaction and further decreased lung damage induced by ventilator-induced lung injury and LPS in rats.\textsuperscript{11–13} However, there are no reports of therapeutic or preventive effectiveness of UTI for radiation-induced lung fibrosis.

Thus, the present study examined whether UTI inhibited the TGF-β signaling pathway or reduced radiation-induced lung fibrosis in C57BL/6J mice, the standard mouse strain for studying the pathophysiology of radiation-induced fibrosis. In addition, the therapeutic potential of UTI was analyzed for prolongation of the lifespan of mice irradiated with a significant dose for inducing lung fibrosis.

MATERIALS AND METHODS

Radiation and UTI administration schedule

Figure 1 shows the experimental protocol. Female, 8-week-old C57/BL6J mice of approximately 20 g in weight were purchased from Charles River Laboratories (Japan) and used for the present study. For the radiation schedule, mice were anesthetized by intraperitoneal injections of Pentobarbital (30 mg/kg) and placed in the supine position for irradiation. The irradiation characteristics were as follows: beam energy: 200 kVp X-ray (Stabilipan 2, Siemens, Germany); dose-rate: 1.47 Gy/minutes; size of the radiation field: semicircle of 2 cm². After irradiation, the mice were maintained with five animals per cage. Treated and control mice were sacrificed by cervical dislocation at time points corresponding to beginning of the fibrotic phase.

In order to analyze the protective effects of UTI on the development of lung fibrosis according to the timing between irradiation and UTI administration, UTI was administrated intraperitoneally at a dose of 200,000 units/kg at 30 minutes before radiation and 60 minutes after radiation for a concurrent group and daily administration during the post-irradiation period of 8 to 14 days for a post-radiation group. The timing of UTI administration for a post-radiation group was determined based on the Rube’s study in which TGF-β mRNA level increased during 8 to 14 days after lung irradiation.\textsuperscript{14} Normal saline was administered to the control group (RT-alone mice).

Irradiated mice untreated (n = 10) or treated with UTI (n = 10) were sacrificed at 16 weeks after irradiation, which corresponds to the pneumatic phase.\textsuperscript{15,16} The whole lungs were removed immediately without being perfused. The left lungs were placed in fixative for histological and immunohistochemical analysis.

Histological analysis

For histological analysis, the left lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at an average thickness of 3 μm. The mounted sections were subjected to hematoxylin and eosin staining. Two blinded observers graded the degree of lung fibrosis independently, according to an established grading scale from 0 (normal lung) to 8 (severe distortion of structure) as shown in Table 1.\textsuperscript{17} The histological features corresponding to the grading scale are shown in Fig. 2. Mean fibrosis score was calculated as the mean grade of 5 microscopic fields in each specimen.

Immunohistochemical staining

Paraffin-embedded tissues were sectioned at 3-μm thickness, deparaffinized, and hydrated using a xylene solution and graded ethanol. Sections were then placed in an antigen retrieval solution (Target Retrieval Solution S1699, DAKO, Denmark). The solution was heated at 95°C for 30 min in a microwave oven and cooled at room temperature for 10 min. After rinsing with deionized water, an endogenous peroxide blocking solution of 0.3% hydrogen peroxide was applied for 30 min at room temperature. The sections were then incubated in 10% goat serum in phosphate-buffered saline (PBS) for 30 min at room temperature to reduce nonspecific antibody binding. Primary anti-TGF-β1 polyclonal antibodies
(sc-146, Santa Cruz, CA, USA) at a dilution of 1:200 were applied overnight in a humidified chamber at 4°C. Slices were washed in PBS and then incubated for 60 min at room temperature with a ready-to-use peroxidase-labeled secondary reagent, ENVISION + TM (K4003 for rabbit antibodies, DAKO, Denmark). After rinsing with PBS, peroxidase activity was visualized using a chromogen mixture (DAB + K3468, DAKO, Denmark). The slides were washed three times for 10 min each in deionized water and counterstained with Mayer’s hematoxylin. Because all of the lung tissue sections were treated in the same way and stained simultaneously under the same conditions, the intensity of immunostaining could be compared. The omission of primary antibodies was used as a negative control and murine bowel tissue as a positive control for TGF-β.

Five fields per lung specimen were examined randomly and more than 500 cells per specimen were counted in order to calculate the rate of TGF-β positive cells. Counts of positively immunostained cells in irradiated lung samples were compared with those in non-irradiated lung at the respective time points.

Statistical methods

The data are presented as means ± SD. Comparisons of the 2 groups were performed using an unpaired Student’s t test. Comparative analysis among the 3 optimization steps was performed using one-way analysis of variance (ANOVA). Post hoc analysis was performed using Tukey’s test. Survival curves were obtained from Kaplan-Meier methods and were compared using the log-rank test. A p value of

<table>
<thead>
<tr>
<th>Grade of fibrosis</th>
<th>Histological features</th>
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<tbody>
<tr>
<td>0</td>
<td>Normal lung</td>
</tr>
<tr>
<td>1</td>
<td>Minimal fibrosis thickening of alveolar or bronchiolar walls</td>
</tr>
<tr>
<td>2</td>
<td>Moderate thickening of walls without obvious damage to lung architecture</td>
</tr>
<tr>
<td>3</td>
<td>Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses</td>
</tr>
<tr>
<td>4</td>
<td>Severe distortion of structure and large fibrous areas; “honeycomb lung” is placed in this category</td>
</tr>
<tr>
<td>8</td>
<td>Total fibrous obliteration of the field</td>
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</tbody>
</table>

Fig. 2. Representative histological features of radiation-induced lung fibrosis. (a) Grade 1 as no fibrosis; (b) grade 3 as minimal; (c) grade 5 as moderate; and (d) grade 7 as severe fibrosis. (Hematoxylin and eosin; × 40 objective).
less than 0.05 was considered as statistically significance. The SPSS software package version 11.0 was used for the analysis.

RESULTS

Histopathologically evaluation

The scores of a non-irradiate control (0 Gy), a small dose (12 Gy), and a large dose (24 Gy) groups were 3.1 ± 1.9, 3.9 ± 0.5, and 6.6 ± 0.2, respectively. There was no statistical difference in the scores between the non-irradiated control group and the small-dose group, but the scores in the large-dose group were significantly increased (p < 0.05).

Figure 3 shows fibrosis scores of irradiated mice treated with UTI concurrently or post-irradiation. Unirradiated control mice showed no significant histopathologic changes; in particular, there was no evidence of pulmonary fibrosis or pneumonitis. Concurrent UTI treatment with irradiation of 24 Gy did not decrease the score of lung fibrosis in RT-alone mice. UTI treatment at 8 to 14 days after irradiation with 24 Gy (post-RT UTI mice) significantly decreased the score of lung fibrosis in RT-alone mice. The mean score of lung fibrosis in post-RT UTI mice was 3.2 ± 1.0, which was significantly smaller than the 6.0 ± 1.3 of RT-alone mice (p < 0.05).

Immunohistochemistry

The representative features of TGF-β expression in RT-

Fig. 3. Fibrosis scores of 24 Gy-irradiated mice (n = 10) according to UTI administration method. Concurrent UTI treatment with irradiation dose not decrease the score of lung fibrosis in mice. UTI treatment 8 to 14 days after irradiation (post-RT) decrease the score of lung fibrosis significantly in irradiated mice. (* indicates p < 0.05).

Fig. 5. The mean rate of TGF-β positive cells in irradiated mice lungs (n = 10). The positive rate in 24 Gy irradiated mice without UTI (RT alone) is significantly higher than in mice with UTI treatment after 24 Gy irradiation (post-RT UTI) and the control without irradiation. (* indicates p < 0.01).

Fig. 4. Immunohistochemical staining of TGF-β in corresponding lung tissue. Positive TGF-β expression is stained in the nucleus and/or cytoplasm of bronchiolar epithelial cells. (a) Control without irradiation. (b) Irradiation with 24 Gy (RT alone) increase TGF-β-positive cells. (c) UTI treatment after irradiation (post-RT UTI) decrease TGF-β positive staining (anti-TGF-β1 polyclonal antibodies; sc-146, Santa Cruz, CA, USA; ×40 objective).
UTI Reduces Radiation Lung Fibrosis

alone mice, post-RT UTI mice, and control mice are shown in Fig. 4. Positive TGF-β expression was identified in the nucleus and/or cytoplasm of bronchiolar epithelial cells. Irradiation with 24 Gy increased TGF-β-positive staining of the lung tissue. However, UTI treatment decreased the TGF-β-positive staining of the lung tissue in irradiated mice.

Figure 5 shows the rate of TGF-β positive cells in the lungs of irradiated mice. The positive rate in post-RT UTI mice was 0.18 ± 0.03 and the corresponding rate in RT-alone mice was 0.23 ± 0.04 (p < 0.01).

Figure 6 shows a significant positive correlation between the TGF-β-positive staining of the lung tissue and fibrosis score for all mice (R^2 = 0.26, p < 0.01). Moreover, histological and immunohistochemical findings showed that UTI treatment decreased radiation-induced lung fibrosis and TGF-β expression.

Survival analysis

The survival curves of post-RT UTI mice (n = 30) and RT-alone mice (n = 10) are shown in Fig. 7. The survival rates at 30 weeks in post-RT UTI mice and RT-alone mice were 33% and 10%, respectively. The mice treated with UTI survived significantly longer than irradiated mice without UTI treatment (p < 0.05).

DISCUSSION

Radiation pneumopathy is the reaction of the normal lung tissue to radiation in various target cells. It starts as an exudative inflammation, with the clinical features of interstitial pneumonia 6 to 12 weeks after irradiation, and proceeds to a productive chronic inflammation lasting several months and terminating in scar formation, called lung fibrosis.19,20 TGF-β is a key cytokine in the regulation and general inhibition of cell growth, plays a role in anti-inflammatory actions, and controls the homeostasis of extracellular matrix.21 Fibroblasts, macrophages, and the lung alveolar epithelium have been proposed as the effectors of TGF-β1 in radiation fibrosis.22,23

In the present study, TGF-β positive cells predominantly appeared in lung tissue irradiated with 24 Gy without UTI. Several studies have shown the relationships between TGF-β and radiation-induced pneumonitis. It was reported that plasma TGF-β1 level was associated with radiation-induced pneumonitis and played an important role as a predictor of radiation pneumonitis,24 and that TGF-β1 induced large increases in type I collagen formation by fibroblasts and other cell types.25 At present three actions of TGF-β, which explain the potential roles of this cytokine in fibrosis, have been described.26 Adenovector-mediated gene transfer of active TGF-β1 induces prolonged severe fibrosis in rat lung.27 There was a highly significant correlation between TGF-β immunoreactivity and radiation injury at 2 and 26 weeks post-irradiation.28

The present study demonstrated that there was a strong correlation between radiation-induced fibrosis score and TGF-β positivity of the lung tissues. In addition, the severity of radiation-induced fibrosis increased with the intensity of TGF-β (Fig. 6). Rube CE et al. reported that, following thoracic irradiation with a single dose of 12 Gy, radiation-induced TGF-β release in lung tissue was already appreciable within the first hours post-irradiation and showed a significant increase after 12 hours; subsequently (48 hours, 72 hours, and 1 week post-irradiation) TGF-β expression declined to basal levels. At the beginning of the pneumatic phase, irradiation-mediated TGF-β levels reached maximal values at 2 and 4 weeks, which showed the most striking increases. Type II pneumocytes and fibroblasts (apart from inflammatory cells) served as important sources of TGF-β expression.29 These results indicate increasing of TGF-β level has an important role for radiation-induced lung fibrosis.
Histologically identifiable radiation-induced lung fibrosis occurs when lung tissues receive more than 12 Gy at single irradiation. In the present study detectable histological changes in the lung were not observed at 12 Gy and significant histological changes were observed at 24 Gy. Down JD et al. first reported strain-dependent differences in radiation-induced lung fibrosis by a comparative study on CBA, C57BL, and F1 hybrid cross (CBBF1) mice after right hemithorax irradiation using doses ranging from 15 to 35 Gy. The strain-dependent differences were further analyzed in detail and the association of active TGF-β with fibroblasts might be a characteristic of the initiation of fibrosis in this model. The differences in the levels of TGF-β mRNA may also lead to strain-dependent variation in fibrotic response.

The protease inhibitor UTI has been widely used as a drug for patients with acute inflammatory disorders such as disseminated intravascular coagulation, shock, and pancreatitis. UTI also decreases lung damage induced by ventilator-induced lung injury and LPS in rats. A summary of the current results that the administration of UTI significantly suppressed the radiation-induced lung fibrosis was presented previously. Bao P et al. confirmed the current result but not analyze the long-term survival effect of UTI.

In addition, the administration of UTI at 8 to 14 days after RT suppressed lung fibrosis significantly, but concurrent administration of UTI did not. The present study clearly indicated that 1) UTI administration several days after irradiation could have a prophylactic effect for the prevention of lung fibrosis, and 2) the injection timing of UTI for irradiation was important. It is noteworthy that the UTI administration period, which caused positive suppression of lung fibrosis, corresponded to the period of the increase in TGF-β level by irradiation, and especially before reaching the peak level of TGF-β. Therefore, it was suggested that the suppression of lung fibrosis might be due to the suppression of TGF-β by UTI. Further study is required to determine whether the administration of UTI after the peak level of TGF-β is reached, coincident with 2 to 4 weeks, to suppress lung fibrosis or not.

In the present study, we studied histological and immunohistological analysis at the 16 weeks after the irradiation to lungs. In several in vivo experiments, TGF-β1 levels increased significantly after the irradiation of mice lungs and the suppression of TGF-β induced decreases fibrosis in mice after irradiation. In addition, an in vivo study reported that the inhibition of TGF-β1 receptor decreased bleomycin-induced lung fibrosis in mice. TGF-β increased significantly with evidence of lung fibrosis at 16 weeks after radiation therapy. Therefore, the present UTI suppression study was undertaken 16 weeks after radiation and demonstrated that administration of UTI significantly suppressed TGF-β positive cells. The results of the present study including other reports indicated a positive correlation between TGF-β and lung fibrosis, suggesting that significant suppression of TGF-β by UTI subsequently reduced the grade of manifestation of radiation-induced lung fibrosis. Figure 7, in which mice whose TGF-β scores were lowered by UTI administration had lower lung fibrosis score, but control mice had significant lung fibrosis, confirmed the proposed mechanism.

A positive correlation was observed between the suppression of lung fibrosis by TGF-β inhibition with caffeic acid phenethyl ester. Angelica sinensis, which is popular traditional medicine in China for gynecological diseases, down-regulates hydroxyproline and TGF-β1, and protects mice from radiation-induced pulmonary fibrosis. In these studies, however, the efficacy for prolongation of survival of the mice was not analyzed and it was unclear whether the modification of lung fibrosis with these drugs can contribute to the prolongation of life span or not. Here we first demonstrated that UTI prolonged the survival of mice irradiated with a high-level dose to induce lung fibrosis. UTI may be useful for preventing lung fibrosis for high risk patients following severe radiation.

Other than the inhibition of radiation-induced lung fibrosis, UTI may possess other anti-tumor effects. UTI may inhibit soluble and tumor cell receptor-bound plasmin and inhibit subsequent effects on tumor cell invasion and metastasis. Thus, UTI may be a useful drug for radiation therapy for thoracic cancer in terms of preventing lung fibrosis and also for increasing anti-tumor effects for some cancers.

In conclusion, the present study demonstrated that the administration of UTI after lung irradiation suppressed TGF-β expression and reduced radiation-induced lung fibrosis. In addition, the administration of UTI significantly prolonged the survival of irradiated mice. UTI may prevent lung fibrosis and contribute to the improvement of clinical outcome in the radiation therapy for thoracic malignancies.

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