A p38 MAPK inhibitor, FR-167653, ameliorates murine bleomycin-induced pulmonary fibrosis

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A number of investigations about pulmonary fibrosis have indicated that sustained and augmented expression of some cytokines in the lung are relevant to recruitment of inflammatory cells and accumulation of extracellular matrix components followed by remodeling of the lung architecture (8, 22, 27, 32, 58, 59). In particular, proinflammatory cytokines such as tumor necrosis factor (TNF)-α (35, 36, 39) and interleukin (IL)-1β (40, 48) were demonstrated to play major roles in the formation of pneumonitis and pulmonary fibrosis. However, the mechanisms of production of the proinflammatory cytokines and activation of intracellular signaling cascades triggered by the proinflammatory cytokines are not completely elucidated in pulmonary fibrosis, even in the animal model of pulmonary fibrosis induced by bleomycin.

The signal transduction pathways through the mitogen-activated protein kinases (MAPK) are major pathways by which extracellular stimuli are transmitted to the intracellular signal. Extracellular stimuli such as oxidative stress, osmotic stress (30), heat shock (42), ultraviolet irradiation (21), ischemia-reperfusion (55), lipopolysaccharide (18), proinflammatory cytokines (5, 41), DNA-damaging agents (37), and so on were demonstrated to phosphorylate and activate p38 MAPK and c-Jun NH2-terminal kinase in various types of cells. A synthesized low-molecular-weight pyrazolotriazine derivative, FR-167653, is a potent suppressor of TNF-α and IL-1β production via specific inhibition of p38 MAPK activity (24, 47, 52). Administration of FR-167653 has been shown to exert beneficial effects in animal models of disseminated intravascular coagulation (51), endotoxin-induced shock (52), pulmonary ischemia-reperfusion injury (23), Helicobacter pylori-induced gastritis (47), and glomerulonephritis (49). Because effective therapy has not been established for pulmonary fibrosis, development of an antifibrotic compound is urgently needed. From this viewpoint, it is worthwhile to assess the anti-inflammatory and anti-fibrotic effects of FR-167653. In this investigation, we

PULMONARY FIBROSIS, which is a heterogeneous group of conditions, is characterized by alveolitis, comprising an inflammatory cellular infiltration into the alveolar septae with resulting fibrosis. Idiopathic pulmonary fibrosis (IPF), whose etiology is unknown, is one of clinically important types of pulmonary fibrosis, and the effects of IPF are chronic, progressive, and often fatal. Previous clinical investigations suggested that the prognosis of IPF is poor with a median survival of 2–2.8 yr (6, 34). However, the pathophysiology of pulmonary fibrosis has not been fully understood.

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administered FR-167653 to a murine model of bleomycin-induced pulmonary fibrosis and assessed histopathological changes and effects on cytokine production.

MATERIALS AND METHODS

Reagents. FR-167653 provided by Fujisawa Pharmaceutical (Osaka, Japan) was dissolved at 2% concentration in 0.5% methylcellulose solution (Nikken, Osaka, Japan). To obtain the dosage of 50, 100, or 150 mg/kg body weight (BW), 2.5, 5, or 7.5 μg/BW of 2% FR-167653 solution was injected subcutaneously. Bleomycin hydrochloride (Nihonkayaku, Osaka, Japan) was dissolved at the concentration of 0.267 U/ml with sterile 0.9% NaCl (isotonic saline) for intratracheal administration. Injected bleomycin solution volume was 3 μl/BW to obtain the dosage of 0.8 U/kg BW.

Animals. Six-week-old male ICR mice weighing 28–32 g were purchased from Charles River Japan (Yokohama, Japan) and maintained under specific pathogen-free conditions, 12-h light/dark cycles, and constant temperature with food and water ad libitum at the Osaka University animal facility. Animals were allowed to acclimate in facilities for at least 1 wk before any treatments.

Model of bleomycin-induced pulmonary fibrosis and treatment. The animals were anesthetized with intraperitoneal injections of pentobarbital sodium (~1 mg; Dainippon Pharmaceutical, Osaka, Japan). After recognition of anesthesia, we administered the mice bleomycin solutions intratracheally as previously described (12). Briefly, bleomycin solution was injected through the vocal cord into the trachea with a Hamilton syringe. The day of intratracheal injection with bleomycin was determined as day 0. For the experiments to clarify whether FR-167653 modulates bleomycin-induced pulmonary fibrosis, mice were divided into three groups. The first group (the saline + vehicle group) was injected with saline intratracheally in a volume of 3 μl/BW and treated with 0.5% methylcellulose solution as a vehicle. The second group (the BLM + vehicle group) was injected with bleomycin intratracheally and treated with vehicle. The third group (the BLM + FR-167653 group) was injected with bleomycin intratracheally and treated with FR-167653. Vehicle and FR-167653 were subcutaneously administered once a day from day −1 to day 14. The numbers of mice in the respective groups are indicated in the figures.

Western blot analysis. The mice were killed by inhalation of sevoﬂurane (Maruishi Pharmaceutical, Osaka, Japan) on days 1, 4, 7, or 21 of bleomycin-induced pulmonary fibrosis. After tracheal cannulation by a 24-gauge flexible needle, lungs of the mice were lavaged three times using 1 ml of sterile saline in each wash. Bronchoalveolar lavage fluid (BALF) cells (2 × 10⁶ cells for Western blot analysis of p38 MAPK) were washed with phosphate-buffered saline, resuspended with sample buffer (62.5 mM Tris-HCl, pH 7.4, 2% SDS, 50 mM dithiothreitol, 10% glycerol, and 0.1% bromphenol blue), and boiled for 10 min. The samples were separated by SDS-PAGE, and the separated proteins were transferred to a polyvinylidene ﬂuoride membrane (Immobilon; Millipore, Bedford, MA). Blots were incubated with rabbit primary antibodies against phosphorylated p38 MAPK (New England Biolabs, Beverly, MA), and total p38 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:500 in blocking solution for 1 h at room temperature after incubation for 1 h at room temperature with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) at a at 1:5,000 dilution. Proteins were visualized after incubation of the blots in Western blot chemiluminescence reagent (NEN, Boston, MA) according to the manufacturer’s protocol and exposure to Fuji RXU film (Fujifilm, Minamiashigara, Japan). For Western blot analysis of activating transcription factor (ATF)-2, 6 × 10⁶ BALF cells from the saline + vehicle group, the BLM + vehicle group, and the BLM + FR-167653 group (FR-167653; 150 mg/kg BW) on day 4 (all groups contain 3 mice) were used and lysed with lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Immunoblottings were performed with antibodies against phosphorylated ATF-2 and total ATF-2 (New England Biolabs). The ratio of phosphorylated ATF-2:total ATF-2 was determined by NIH Image analysis.

ELISA of TNF-α. In addition to the bleomycin intratracheal model, we prepared the model that was administered bleomycin intravenously (bleomycin iv model). Mice were divided into five groups (each group contains 3 or 4 mice). The first group was pretreated with 5 μg/BW of 0.5% methylcellulose as a vehicle 2 h before a single intravenous injection of 8 μl/BW saline (saline + vehicle). The second group was pretreated with 5 μg/BW of 0.5% methylcellulose 2 h before a single intravenous injection of 8 μl/BW of 12.5 U/ml bleomycin solution in sterile saline (the dose of bleomycin was 100 U/kg BW; BLM + vehicle). The other groups were pretreated with 2.5, 5, and 7.5 μg/BW of 2% FR-167653 solution 2 h before administration of 100 U/kg BW bleomycin (BLM + FR-167653; 50, 100, and 150 mg/kg, respectively). Two hours after intravenous injection of saline or bleomycin, mice in each group were killed and blood was obtained from the right ventricles. After centrifugation of the blood, the sera were stored at −20°C. Mouse serum levels of TNF-α were measured using the mouse TNF-α ELISA kit (R&D Systems, Minneapolis, MN) with a detection limit of 5.1 pg/ml.

Hydroxyproline analysis. Total collagen content of the whole lungs was estimated by an assay of hydroxyproline (HOP) (50). Briefly, whole lungs were obtained 21 days after bleomycin administration and hydrolyzed with 2 ml of 6 N HCl at 105°C for 16 h in sealed glass tubes (Iwaki, Tokyo, Japan). HOP in the hydrolysate was determined colorimetrically at 562 nm with p-dimethylaminobenzaldehyde (Sigma, St. Louis, MO). HOP (Sigma) concentrations from 0 to 10 μg/ml were used to construct a standard curve. Values are expressed as micromolar of HOP per pair of lungs. The effect of FR-167653 on the HOP content in bleomycin-induced pulmonary fibrosis is indicated as “% reduction,” which was calculated by the following method: % reduction = (HOP of the BLM + vehicle group – HOP of the BLM + FR-167653 group)/HOP of the BLM + vehicle group – HOP of the saline + vehicle group).

Histological examination. On day 21 of bleomycin-induced pulmonary fibrosis, 1 ml of 10% buffered formaldehyde was instilled to the lung via an intratracheal cannula, and the whole lungs were excised and fixed in 10% buffered formaldehyde in preparation for histological examination (inflation fixation method). The fixed lungs were embedded in paraffin, sectioned sagittally, and stained with hematoxylin-eosin. For the quantitative histological analysis of fibrotic changes induced by bleomycin, a numerical fibrotic scale (Ashcroft score) (3) was used. The Ashcroft score was obtained as follows. The severity of the fibrotic changes in each lung section was assessed as a mean score of severity from the observed microscopic fields. More than 40 fields covered each whole lung section, observed at a magnification of ×100 in each field, and the severity of the fibrotic changes in each field was assessed and allotted a score from 0 (normal) to 8.
Phosphorylation of p38 MAPK and ATF-2 in BALF cells after intratracheal administration of bleomycin.

We tested phosphorylation of p38 MAPK and ATF-2, one of the substrates of p38 MAPK, in BALF cells by intratracheal administration of bleomycin (Fig. 1). In BALF cells from no-treatment control mouse, basal level of phosphorylated p38 MAPK (Fig. 1A, top, no-treat lane) was detected. The samples of the saline + vehicle group on day 4 and day 7 (saline intratracheal on day 0, vehicle subcutaneous on day −1 to day 3 or day 6) were also tested, and they had the same intensity as the sample of the nontreated mouse (data not shown). The level of phosphorylated p38 MAPK of the BLM + vehicle group was markedly augmented one day after intratracheal administration of bleomycin (0.8 U/kg BW), and the augmentation lasted for at least 21 days. Also, bleomycin exposure augmented phosphorylation of ATF-2 of BALF cells on day 4 (Fig. 1, B and C; \( P = 0.0068 \)). The samples of the nontreated mouse had the same intensity as phosphorylated ATF-2 of the saline + vehicle group on day 4 (data not shown). Next, we tested the in vivo effect of FR-167653 on phosphorylation of ATF-2. The mice were administered with bleomycin on day 0, and then 150 mg/kg BW FR-167653 was subcutaneously injected from day −1 to day 3. The BALF cells were harvested on day 4 of bleomycin treatment. As shown in Fig. 1, B and C, phosphorylation of ATF-2 in the BALF cells was significantly inhibited by the FR-167653 treatment (\( P = 0.014 \)). Equal loading was checked using an antibody against total p38 MAPK and total ATF-2 (Fig. 1A, bottom; Fig. 1B, bottom).
Effect of FR-167653 on TNF-α/H9251 expression in sera after intravenous injection of bleomycin. We elucidated the effect of FR-167653 on TNF-α/H9251 expression in sera after intravenous injection of bleomycin. Vehicle or FR-167653 (50, 100, and 150 mg/kg BW) was subcutaneously administered 2 h before bleomycin (100 U/kg BW) intravenous injection. Two hours after the bleomycin injection, serum levels of TNF-α/H9251 were determined by ELISA (Fig. 2). In the sera from mice that were pretreated with vehicle and injected with bleomycin (BLM + vehicle), significant amounts of TNF-α (75.77 ± 11.13 pg/ml) were detected. The augmentation of TNF-α expression was significantly reduced to nearly the basal level by the pretreatment of 100 or 150 mg/kg of FR-167653 (*P < 0.0001). In the sera of the mice instilled with saline instead of bleomycin, less than the detection limit of TNF-α was detected.

Effect of FR-167653 on HOP content of bleomycin-induced lung fibrosis. The effect of FR-167653 on pulmonary fibrosis 21 days after bleomycin intratracheal administration (0.8 U/kg BW) was assessed by evaluation of the HOP content of the whole lungs (Fig. 3). The HOP content of the lungs of the BLM + vehicle group increased approximately twofold compared with that of the saline + vehicle group in these experiments. The HOP contents of the BLM + vehicle group were not significantly different from the lungs instilled with bleomycin alone (not shown). The treatment with 50, 100, and 150 mg/kg BW of FR-167653 exerted 31.6, 66.1, and 61.9% reduction of the HOP contents compared with treatment with the vehicle, respectively (**P < 0.0005, and ***P < 0.0001; the BLM + vehicle group vs. the BLM + FR-167653 group, respectively). Subsequent experiments were examined with the treatment of 150 mg/kg BW of FR-167653.

Effect of FR-167653 on histopathological changes of bleomycin-induced pulmonary fibrosis. The effect of FR-167653 on bleomycin-induced pulmonary fibrosis on day 21 was also tested histopathologically. The lung sections of the BLM + vehicle group showed marked histopathological changes, such as large fibrous areas, collapsed alveolar spaces, and traction bronchiectasis in the subpleural and peribronchial regions (Fig. 4B). The histopathological characteristics of the BLM + vehicle group were not significantly different from the lungs instilled with bleomycin alone (not shown). How-

Fig. 2. Effect of FR-167653 on tumor necrosis factor (TNF)-α expression in sera after intravenous injection of bleomycin. The saline + vehicle group was pretreated with 0.5% methylcellulose as a vehicle 2 h before a single intravenous (iv) injection of saline. The BLM + vehicle group was pretreated with the vehicle 2 h before a single iv injection of bleomycin (100 U/kg BW). The BLM + FR-167653 group was pretreated with 50, 100, and 150 mg/kg BW of FR-167653 2 h before a single iv injection of bleomycin (100 U/kg BW). Two hours after iv injection of saline or bleomycin, blood was obtained from the right ventricles. Bars denote the means ± SE of the serum TNF-α concentration (conc.) (picograms/milliliters) of each experimental group. The P values compared with the value of the BLM + vehicle group are described as *P < 0.01.

Fig. 3. Effect of FR-167653 on hydroxyproline (HOP) content of bleomycin-induced pulmonary fibrosis. HOP content in whole lungs on day 21 was estimated as described in MATERIALS AND METHODS. Open bars, saline intratracheally on day 0, vehicle subcutaneously on day –1 to day 14; hatched bars, bleomycin 0.8 U/kg BW intratracheally on day 0, vehicle subcutaneously on day –1 to day 14; closed bars, bleomycin 0.8 U/kg BW intratracheally on day 0, 50 mg/kg BW of FR-167653 (A), 100 mg/kg BW (B), and 150 mg/kg BW (C) subcutaneously on day –1 to day 14. Values are means ± SE.
ever, in the lungs from the BLM + FR-167653 group (150 mg/kg BW of FR-167653; Fig. 4C), fibrotic lesions were observed, but its extent was limited and its intensity was attenuated compared with the BLM + vehicle group. As shown in Fig. 4A, the control lung instilled with saline (the saline + vehicle group) showed no histopathological change. To confirm the effect of FR-167653 on the histopathological change of bleomycin-induced pulmonary fibrosis, the overall grades of the fibrotic changes of the lungs were obtained by numerical score (Ashcroft score) on day 21 (Fig. 4D). The score of the BLM + FR-167653 group (2.62 ± 0.22) was significantly suppressed compared with the BLM + vehicle group (4.72 ± 0.26; P = 0.0001).

**TUNEL analysis of the lungs on day 9 of bleomycin-induced pulmonary fibrosis.** We tested the effect of FR-167653 (150 mg/kg BW) on apoptosis of lung cells induced by bleomycin instillation. To assess apoptosis, we performed TUNEL staining on the sections of the lungs on day 9 of bleomycin-induced pulmonary fibrosis and searched TUNEL-positive signals at a magnification of ×400. The signals of TUNEL stain were detected at the inflammatory and the fibrotic foci more abundantly in the BLM + vehicle group than in the BLM + FR-167653 group, where only a small number of apoptotic cells were detected. Figure 5A shows the control lung of the saline + vehicle group, which also shows few apoptotic cells. The numbers of the signals in total fields of the lungs were counted at a magnification of ×100 (Fig. 5A). The mean positive cell number per ×100 field was significantly decreased in the BLM + FR-167653 group (9.95 ± 2.47) compared with the BLM + vehicle group (27.75 ± 6.83; P = 0.026; Fig. 5D). The saline + vehicle group had few apoptotic cells (4.56 ± 1.27).

**Effect of FR-167653 on TNF-α, mCTGF, and TGF-β mRNA expressions of the whole lungs of bleomycin-induced pulmonary fibrosis.** To further elucidate the effect of FR-167653 on lung inflammation and also on fibrosis, we assessed TNF-α, mCTGF, and TGF-β mRNA expression in the lung (Fig. 6). The lungs were serially obtained from mice instilled with bleomycin and treated with vehicle or 150 mg/kg BW of FR-167653. In the lungs of the BLM + vehicle group, increased TNF-α mRNA expression was detected on day 4 and day 7 of bleomycin-induced pulmonary fibrosis, and the augmented expression returned to the basal level by day 14. However, the augmentation of TNF-α mRNA expression of the BLM + vehicle group was markedly suppressed by the FR-167653 treatment on day 4 and day 7. By the densitometric analysis, TNF-α mRNA expression on day 4 of the BLM + FR-167653 group was statistically lower than the BLM + vehicle group (Fig. 6B, P = 0.0055).

Profibrotic cytokines such as mCTGF and TGF-β were also assessed. In the BLM + vehicle group, mCTGF mRNA expression showed peaks on day 7 and day 14. The FR-167653 treatment markedly suppressed the mCTGF mRNA expression on day 14 (Fig. 6C, P = 0.0004). Increase of TGF-β mRNA expression was detected in both of the mice treated with the vehicle and FR-167653, but no definite difference was seen between them.

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**Fig. 4.** Effect of FR-167653 on histopathological changes of bleomycin-induced pulmonary fibrosis. Mice were treated as described in Fig. 3. The lungs of the mice on day 21 were fixed in 10% formaldehyde, routinely processed, and stained with hematoxylin-eosin. A: saline + vehicle group (saline intratracheally on day 0, vehicle subcutaneously on day –1 to day 14). B: BLM + vehicle group (bleomycin 0.8 U/kg BW it on day 0, vehicle sc on day –1 to day 14). C: BLM + FR-167653 group (bleomycin 0.8 U/kg BW it on day 0, 150 mg/kg BW of FR-167653 sc on day –1 to day 14). The left lungs are shown in A–C. Original magnification, ×4. The lung sections of the BLM + vehicle group show marked histopathological changes such as large fibrous areas, collapsed alveolar spaces, and traction bronchiectasis (B). However, in the lungs from the BLM + FR-167653 group (C), fibrotic lesions are observed in the peribronchial area, but its extent is limited and its intensity is attenuated compared with the vehicle-treated mice. D: evaluation of fibrotic changes on day 21 in the lung by numerical fibrotic score (Ashcroft score). Bars denote Ashcroft scores (means ± SE) of each experimental group.
p38 MAPK played a critical role in the pathogenesis of pulmonary fibrosis. These observations strongly suggest that p38 MAPK is one of the signal transducers to contribute to the formation of bleomycin-induced pulmonary fibrosis. Research for other pathways except for p38 MAPK may be relevant to suppression of TNF-α and CTGF expression. Another important characteristic was that FR-167653 suppressed the fibrosis formation induced by bleomycin without any marked adverse effects. These observations suggest that p38 MAPK is an important target of regulation of pulmonary fibrosis, and FR-167653 may be a feasible novel therapeutic agent.

**Effect of FR-167653 on weight gain of mice.** To test whether FR-167653 ameliorates pulmonary cachexia of bleomycin-induced pulmonary fibrosis, we compared the weight gain of mice in each group (Fig. 7). Before instillation of bleomycin to a mouse on day 0, each mouse was weighed. On day 21, the mice were weighed again before being killed. The difference in BW between day 0 and day 21 was determined as the increase in BW. In the 3 wk of the experimental course, mice of the saline + vehicle group gained 11.0 ± 0.57 g BW. The mice of the BLM + vehicle group were cachexic and gained 3.89 ± 0.93 g BW. However, the mice of the BLM + FR-167653 group (150 mg/kg BW of FR-167653) gained significantly more weight (6.50 ± 0.63 g) compared with the BLM + vehicle group (P = 0.028).

**DISCUSSION**

In the present study, we confirmed that bleomycin exposure induces p38 MAPK activation and phosphorylation of ATF-2, a substrate of p38 MAPK, in murine BALF cells, and that the specific inhibitor, FR-167653, inhibited phosphorylation of ATF-2. Moreover, FR-167653 exerted in vivo anti-inflammatory, antifibrotic, and antiapoptotic effects in the mouse model of pulmonary fibrosis induced by bleomycin. These observations strongly suggest that p38 MAPK played a critical role in the pathogenesis of lung fibrosis induced by bleomycin, and the favorable effect of FR-167653 may be relevant to suppression of TNF-α and CTGF expression. Another important characteristic was that FR-167653 suppressed the fibrosis formation induced by bleomycin without any marked adverse effects. These observations suggest that p38 MAPK is an important target of regulation of pulmonary fibrosis, and FR-167653 may be a feasible novel therapeutic agent.

p38 MAPK was demonstrated to be activated by various extracellular stimuli, and the present study revealed that bleomycin also activates p38 MAPK. After bleomycin exposure, p38 MAPK was phosphorylated as early as day 1, and this phosphorylation sustained to day 21. ATF-2 was also phosphorylated by bleomycin exposure. In BALF cells harvested from mice of the BLM + FR-167653 group on day 4, lower amounts of phosphorylated ATF-2 were detected compared with the BLM + vehicle group (2, 9).

The assays of HOP contents indicated a dose-dependent effect of FR-167653 on bleomycin-induced pulmonary fibrosis. This result revealed the important role of p38 MAPK on bleomycin-induced pulmonary fibrosis. However, the inhibitory effect on fibrosis formation at doses of 100 and 150 mg/kg BW did not differ and reduced the ceiling of ~60% reduction. It is natural that p38 MAPK is one of the signal transducers to contribute to the formation of bleomycin-induced pulmonary fibrosis. Research for other pathways except for p38 MAPK may be relevant to suppression of TNF-α and CTGF expression. Another important characteristic was that FR-167653 suppressed the fibrosis formation induced by bleomycin without any marked adverse effects. These observations suggest that p38 MAPK is an important target of regulation of pulmonary fibrosis, and FR-167653 may be a feasible novel therapeutic agent.
p38 MAPK in bleomycin-induced pulmonary fibrosis is needed.

In the pathogenesis of pulmonary fibrosis, TNF-α augments synthesis of fibronectin (4), prostaglandin (15), and TGF-β (38) in vitro. We also observed that the IL-10 gene transfer ameliorated bleomycin-induced pulmonary fibrosis with suppression of TNF-α expression (1). It was also shown that in vivo overexpression of TNF-α in the lung induces pulmonary fibrosis. Sime et al. (45) introduced the TNF-α gene by the replication-deficient adenovirus vector into rat lungs, and they observed 7–10 day persistence of the gene expression. Miyazaki et al. (29) also showed that lung-specific expression of TNF-α in transgenic mice results in the development of chronic inflammation and severe fibrosis of the lungs. In the present study, TNF-α was detected in the sera of the mice 2 h after intravenous injection of bleomycin, and FR-167653 suppressed serum TNF-α concentration. The present finding regarding the immediate induction of TNF-α is compatible with previous observations (46), and the present findings clearly demonstrate that induction of TNF-α by intravenous administration of bleomycin was mediated by activation of p38 MAPK. Moreover, we observed significant suppression of TNF-α mRNA expression in the bleomycin-induced lung fibrosis model by FR-167653. Some previous studies demonstrated that another p38 MAPK inhibitor, SB-203580, reduced lipopolysaccharide-stimulated secretion of TNF-α protein in human monocytes or lymphocytes but did not distinguish between transcriptional, posttranscriptional, or translational modes of action (10, 19, 43).

Profibrotic cytokines such as TGF-β (7, 14, 33) and CTGF (11, 26) also play critical roles in the pathogenesis of pulmonary fibrosis. As shown in Fig. 6, FR-167653 administration significantly suppressed the expression of mCTGF mRNA during the later phase (day 14) of bleomycin instillation. This observation offers a partial explanation for the amelioration of fibrotic indices assessed on day 21.

In the present investigation, FR-167653 did not affect TGF-β mRNA expression. It has been reported that posttranslational activation is more critical than the amount of TGF-β mRNA. The latent form of TGF-β
interacts with αvβ6-integrin (31) or thrombospondin-1 (53), and activation ensues by removal of latency-associated peptide from the NH2 terminus of the latent form (54). Hence, the TGF-β mRNA level may not necessarily reflect the TGF-β activity, and this finding did not completely negate the relevance of TGF-β to lung fibrosis induced by bleomycin. Moreover, because the level of CTGF expression was demonstrated to be upregulated only by TGF-β (20), the suppressed CTGF expression suggested that TGF-β activity might be decreased in the lungs of the BLM + FR-167653 group. It was reported that, in the lungs after bleomycin administration, apoptosis of bronchial and alveolar epithelial cells is observed (17, 25). Moreover, it was also reported that apoptosis of pneumocytes induced by agonistic antibody against Fas results in lung fibrosis (16). In the present investigation, FR-167653 administration markedly suppressed apoptosis of the lung cells. The role of p38 MAPK on apoptosis of various cell types is controversial, but it is possible that the suppression of apoptosis by FR-167653 is at least explained by inhibiting death signals transduced by Fas ligand and TNF-α whose expressions were enhanced by p38 MAPK (57).

As shown in Fig. 7, bleomycin reduced the BW gain by inducing pulmonary cachexia. This toxicity of bleomycin assessed by BW gain was partially reversed by FR-167653 administration. This suggested the efficiency of FR-167653 for pulmonary fibrosis. Most investigations assessing the in vivo anti-inflammatory effects of FR-167653 report favorable effects, including amelioration of mortality without marked side effects. However, Gardiner et al. (13) reported that FR-167653 caused increases in plasma creatine kinase and the lactate dehydrogenase level. The toxicity of FR-167653 for primates should be extensively studied, and assessment of its feasibility as an anti-inflammatory agent on COX (47). In this respect, FR-167653 may be superior to SB-203580, which has an inhibitory effect on COX activity.

In conclusion, the present study demonstrates that p38 MAPK inhibitor FR-167653 ameliorates the formation of bleomycin-induced pulmonary fibrosis. The present findings suggest that p38 MAPK is involved in bleomycin-induced pulmonary fibrosis. Accumulation of evidence regarding intracellular signal transduction in pulmonary fibrosis may reveal the mechanism of inflammation accompanied with fibrosis and may help to develop potent drugs for pulmonary fibrosis.

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