Assay of Hydroxyproline

Hydroxyproline in the murine lung was assayed at each time point according to the commonly used procedure of colorimetric measurement by Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan) [36, 37]. Briefly, lungs were harvested on each designated day post bleomycin administration and homogenized in 1 mL of phosphate-buffered saline (PBS), pH 7.4, with a Tissue-Tearor. One-half milliliter of each sample (left lung) was then digested in 1 ml of 6 N HCl for 8 hours at 120°C. Five microliters of citrate-acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 mL of chloramines T solution (282 mg of chloramine T, 2 mL of *n*-propanol, 2 ml of H_2O, and 16 mL of citrate-acetate buffer) were added to 5 mL of sample, and the samples were left at room temperature for 20 minutes. Next, 100 mL of Ehrlich's solution, 9.3 mL of n-propanol, and 3.9 ml of 70% perchloric acid were added to each sample, and the samples were incubated for 15 minutes at 65°C. Samples were cooled for 10 minutes and read at 550 nm on a spectrophotometer. Hydroxyproline concentrations from 0 to 400 mg/mL were utilized to construct a standard curve. The hydroxyproline content of the left lung in each subject represent μg/lung.

Cell Culture

For in vitro experiments, A549 cells (human type II alveolar epithelial cells) and WI38 cells (human lung fibroblasts) were cultured in tissue flasks incubated in 100% humidity and 5% CO_2 at 37°C in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY) and penicillin-streptomycin (50μg/mL, GIBCO BRL), at 1×10^6 cells/mL. A549 cells and WI38 cells were then plated onto 6-well, flat-bottom tissue culture plates (Becton, Dickinson, Franklin Lakes, NJ) at a density of 2×10^5 cells/well in RPMI 1640 medium. The medium was changed every 2nd day until the cells became confluent, and then the cells were used for the experiments. After cultures of A549 and WI38 were grown to reach confluence, the cultures were washed 3 times with 2 mL PBS, followed by the addition of 2 mL fresh serum-free medium (RPMI 1640) containing IL-1β, TNF-α, IL-4, or combinations of these cytokines, and the mixtures were incubated for 36 hours. A549 and WI38 cells were also stimulated with a combination of IL-1β (10 ng/mL), TNF-α (100 ng/mL), and IL-4 (100 ng/mL), and incubated for 36 hours. Cells were lysed and used for isolation of RNA for RT-PCR. Cell-free culture supernatants were collected and assayed for ST2. Human ST2 was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Medical

& Biological Laboratories, Nagoya, Japan) according to the manufacturers' instructions. In brief, samples of culture supernatants or standards were incubated in microwells coated with antihuman ST2 antibody. After microwells were washed, peroxidase-conjugated antihuman ST2 antibody was added into the microwells and the samples were incubated. After another washing, the peroxidase substrate was added and the optical density at 450 nm was determined. All samples were assayed in duplicate.

Analysis of mRNA Expression for ST2, Cytokines, and Growth Factor by RT-PCR

Total RNA was extracted from whole lung tissue and cultured cells using Trizol reagent (Life Technologies, Rockville, MD). Five hundred nanograms of RNA were reverse-transcribed and amplified by RT-PCR using an RT-PCR kit (Takara, Tsukuba, Japan) under the following conditions: 94°C, 1 minute; 57°C, 1 minute; and 72°C, 1.5 minutes for each cycle as previously described [25]. The sequences of primers are shown in Table 1. The optimal numbers of PCR cycles were as follows: 34 for β-actin, IL-1β, and TNF-α; 38 for ST2, transforming growth factor (TGF)-β1, and IFN-γ; 42 for IL-4 and IL-5. The PCR products of murine IFN-γ, IL-4, IL-5, ST2, IL-1β, TNF-α, TGF-β1, and β-actin were fragments of

TABLE 1 The Sequence of Primers

Gene	Primers	Product size	PCR cycles	Species
IFN-γ	F: CTGTTTCTGGCTGTTACTGC R: TCAGCAGCGACTCCTTTTCC	426 bp	38	Murine
IL-4	F: CATCCTGCTCTTCTTTCTCG R: GATGCTCTTTAGGCTTTCCAG	378 bp	42	Murine
IL-5	F: ACAAGCAATGAGACGATGACG R: GCCTTCCATTGCCCACTCT	272 bp	42	Murine
ST2	F: ACTTCTTGGCTGATGTCCTG R: CAATGTGTGAGGGACACTCC	266 bp	38	Murine
IL-1β	F: AAGTGTCTGTCGAC R: AGTCCTCGAGGTACC	447 bp	34	Murine
TNF-α	F: TCTCAGCCTCTTCTCATTCC R: GAACCTGGGAGTAGACAAG	331 bp	34	Murine
TGF-β1	F: CGGACTACTATGCTAAAGAGG R: GTATTCCGTCTCCTTGGTTC	344 bp	34	Murine
β-Actin	F: TGTCCCTGTATGCCTCTGGT R: ACTGTGTTGGCATAGAGGTC	477 bp	34	Murine
ST2	F: AGGCTTTTCTCTGTTTCCAGTAATCGG R: CAGTGACACAGAGGGAGTTCATAAAGTTAGA	659 bp	38	Human
β-Actin	F: CTCGTCATACTCCTGCTTGC R: GGGACCTGACTGACTACC	547 bp	38	Human

F = sense primer.
R = antisense primer.

426, 378, 272, 266, 447, 331, 222, 344, and 477 bp in length, respectively, and the PCR products of human ST2 and β-actin were fragments of 659 and 547 bp in length, respectively (Table 1). The amplified products were electrophoresed on 3.0% agarose gel, and the intensity of DNA bands was quantified by densitometry (National Institutes of Health, IMAGE 1.61) and was normalized to β-actin expression. The cytokine to β-actin ratio of each subject was then standardized by the mean of that in baseline level (untreated mice or serum-free control medium).

Statistical Analysis

Data were expressed as the mean ± SEM. Values were compared with baseline levels determined in untreated animals (day 0) or serum-free control medium. Multiple comparisons were carried out by the Fisher protected least-significant differences method followed by the post hoc test. Analysis of correlation was done with the Pearson coefficient of correlation. $P < .05$ was considered statistically significant.

RESULTS

Lung Hydroxyproline Levels of the BLM-Induced Pulmonary Fibrosis Model

The hydroxyproline content had gradually increased and was significantly higher at days 7, 14, 21, 28, and 56 after BLM instillation compared to untreated mice (Table 2). Although the numbers of samples at each time point were 4, similar results were obtained in 3 separate experiments. These data suggest that significant accumulation of collagen in the lungs started at day 7 after BLM instillation in our model.

TABLE 2 Kinetics of the Hydroxyproline Content in the Left Lung of the BLM-Induced Pulmonary Fibrosis Model

Time (days)	Hydroxyproline content (μg/left lung)
0	50.5 ± 5.6
1	66.4 ± 2.7
4	66.6 ± 2.4
7	82.3 ± 7.4*
14	98.0 ± 13.1*
21	91.6 ± 11.7*
28	101.1 ± 13.4*
56	121.4 ± 10.3*

*$P < .05$ versus baseline levels (day 0).

ST2, Cytokines, and TGF-β1 Expression in a BLM-Induced Pulmonary Fibrosis Model

We analyzed the kinetics of ST2, cytokines, and TGF-β1 mRNA expression in lungs after BLM exposure by RT-PCR. Inducible expression of ST2, IL-4, IL-5, IL-1β, TNF-α, and TGF-β1 mRNA in the lung were observed after post BLM inoculation, whereas IFN-γ mRNA expression in the lung was slightly decreased (Figure 1*A*). The IFN-γ/β-actin mRNA ratio was slightly reduced on day 7, but not significantly (Figure 1*C*). On the other hand, lung IL-4 and IL-5 mRNA expression were just barely detectable in untreated mice, but those were significantly up-regulated after BLM treatment (Figure 1*A*). The increases in the lung IL-4/β-actin and IL-5/β-actin mRNA ratio were statistically significant on day 7 and on days 4, 7, 14, and 28 after BLM treatment, respectively, compared with baseline

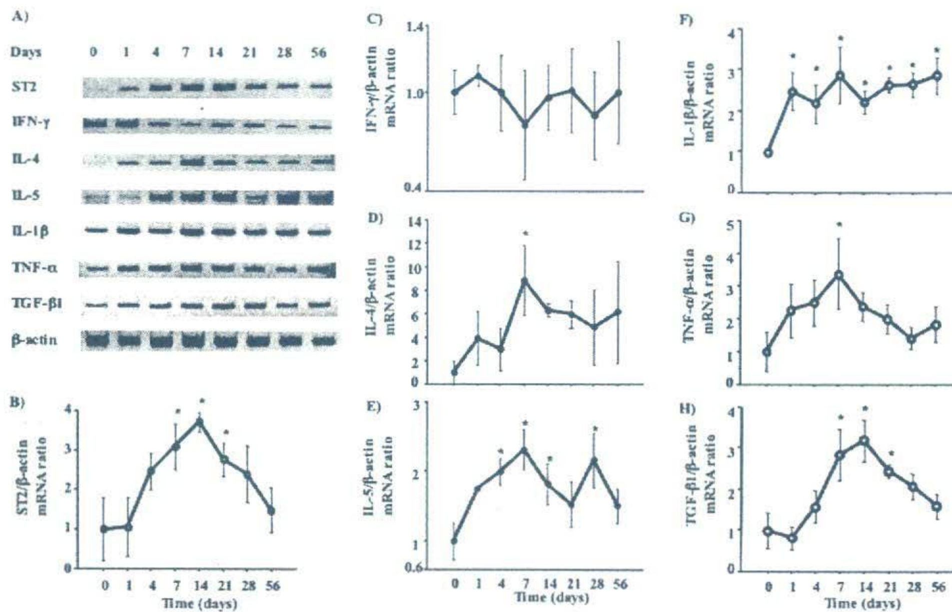

FIGURE 1 Kinetics of ST2, TGF-β1, or cytokine mRNA expression of the lung tissue in the BLM-induced pulmonary fibrosis model. (*A*) Total RNA was extracted from the lung at the indicated time before (day 0) and after intratracheal administration of BLM as described in Materials and Methods. The cDNA samples obtained by RT-PCR for ST2, IFN-γ, IL-4, IL-5, IL-1β, TNF-α, TGF-β1, or β-actin were loaded on a 3.0% agarose gel. The data shown are representative of 4 independent experiments. (*B–H*) Densitometric analyses of ST2 (*B*), IFN-γ (*C*), IL-4 (*D*), IL-5 (*E*), IL-1β (*F*), TNF-α (*G*), and TGF-β1 (*H*) mRNA expression in the lung tissues were carried out. The relative levels designate the ratio obtained from the density of the band of ST2, TGF-β1, or cytokines mRNA expression were normalized to β-actin expression, and then those to the β-actin ratio of each subject were standardized by the mean of that in untreated mice. Data are presented as mean relative densities ± SEM (n = 4 in each group). $^{*}P < .05$ versus untreated mice (day 0).

levels (Figure 1*D*, *E*). Furthermore, both IL-1β and TNF-α mRNA expression in the lung also increased and reached a peak at day 7 (Figure 1*F*, *G*). These data of the kinetics of gene expression in the BLM-treated lung demonstrated that Th2-type cytokines and proinflammatory cytokines were significantly up-regulated on day 7, which indicates the time of the beginning of lung fibrosis (Table 2). In the lungs, very weak expression of ST2 mRNA expression could be detected before BLM instillation (day 0), but expression appeared after BLM instillation and increased significantly between days 7 and 21, which declined to the baseline levels after day 56 (Figure 1*A*, *B*). ST2 mRNA expression in the lung reached a peak at day 14, approximately 3.7-fold higher than the baseline level (Figure 1*B*). Lung TGF-β1 mRNA expression increased significantly between days 7 and 21, and reached a peak at day 14 after BLM instillation (Figure 1*H*).

Correlation of ST2 Gene Expression to Cytokines Expression, TGF-β1 Expression, and Hydroxyproline Levels in the Lung of the BLM-Induced Pulmonary Fibrosis Model

TGF-β is a well-known stimulant of extracellular matrix production by fibroblasts and has been suggested to play an important role in the development of pulmonary fibrosis [38]. Interestingly, the kinetics of ST2 gene expression shows a similar trend to TGF-β1 gene expression (Figure 1*B*, *H*). To evaluate the relationship between ST2 and cytokines, TGF-β1, or hydroxyproline, we analyzed the factors associated with ST2 mRNA expression in the lung of the BLM-induced pulmonary fibrosis model using Pearson's correlation coefficient test. The ST2 mRNA expression statistically correlated with hydroxyproline levels ($r = .360$, $P = .042$), IL-5 ($r = .531$, $P = .001$), and TGF-β1 ($r = .437$, $P = .012$) mRNA expression in the BLM-treated lung (Table 3). Other factors, such as IFN-γ, IL-4, IL-1β, and TNF-α mRNA expression, did not correlate with the ST2 mRNA

TABLE 3 Correlations among ST2 mRNA Expression, Other Cytokine mRNA Expression and Hydroxyproline Levels in the Lung of BLM-Induced Pulmonary Fibrosis Model

	ST2 mRNA	
	r	*P* value
IFN-γ mRNA	0.300	0.095
IL-4 mRNA	0.201	0.272
IL-5 mRNA	0.531	0.001*
IL-1β mRNA	0.060	0.732
TNF-α mRNA	−0.097	0.602
TGF-β1 mRNA	0.437	0.012*
Hydroxyproline level	0.360	0.042*

expression (Table 3). These data suggest that ST2 might be closely related to the fibrotic process in the lung of the BLM-induced pulmonary fibrosis model.

Synergistic Effects of IL-1β, TNF-α, and IL-4 on ST2 Expression from A549 and WI38 Cells

The kinetics of gene expression in the lung of the BLM-induced pulmonary fibrosis model demonstrated that the peak in gene expression of Th2-type cytokines (IL-4 and IL-5) and proinflammatory cytokines (IL-1β and TNF-α) occurred earlier than the peak in ST2 and TGF-β1 expression (Figure 1). We therefore hypothesized that the expression of ST2 may be up-regulated, reflecting an inflammatory response induced by the combination of proinflammatory cytokine and Th2-type cytokine in this model.

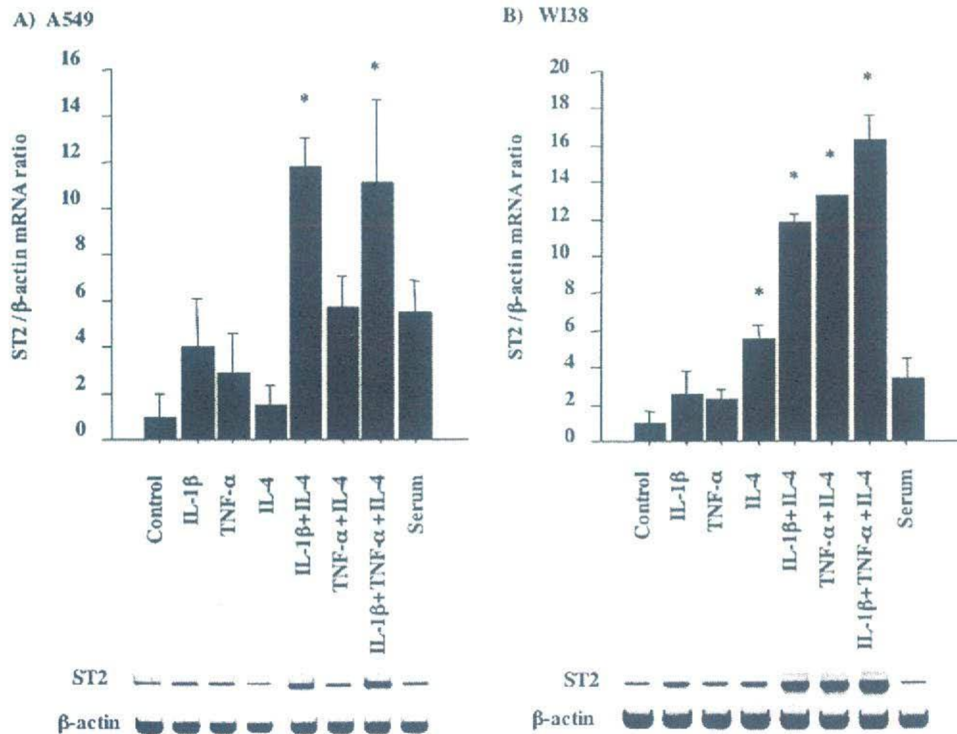

FIGURE 2 Additive effect of IL-1β, TNF-α, and IL-4 on the increase of ST2 mRNA expression in A549 and WI38 cells. A549 (*A*) and WI38 (*B*) cells were stimulated with IL-1β (10 ng/mL), TNF-α (100 ng/mL), IL-4 (100 ng/mL), or a combination of these cytokines for 36 hours. Total RNA was extracted from A549 and WI38 cells, and the cDNA samples obtained by RT-PCR for ST2 or β-actin were loaded on a 3.0% agarose gel. "ST2/β-actin" indicates the ratio obtained from the density of the band of ST2 mRNA expression, standardized with that of β-actin mRNA expression level. The mean relative densities ± SEM are shown for the cumulative data from each of 3 samples in 3 independent experiments. *$P < .05$ versus control (serum-free medium alone).

Reports have demonstrated that the expression of ST2 was enhanced by proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α [33, 34], and Th2 cells cultured with IL-4 led to increased ST2L expression [39]. To our knowledge, there have been no reports describing a synergistic effect of proinflammatory cytokine and Th2-type cytokine in inducing the expression of ST2. It has recently been suggested that IPF involves abnormal wound healing in response to multiple, microscopic sites of ongoing alveolar epithelial injury and activation associated with the formation of patchy fibroblast-myofibroblast foci, which evolve to fibrosis [40]. Therefore, we investigated the capacity of alveolar epithelial cells and fibroblasts to induce ST2 mRNA in response to the combination of proinflammatory cytokine and Th2-type cytokine. We used cell lines A549 and WI38, which were derived from human type II alveolar epithelial cells and human lung fibroblasts, respectively. A549 and WI38 cells were exposed to IL-1β (10 ng/mL), TNF-α (100 ng/mL), IL-4 (100 ng/mL), or a combination of

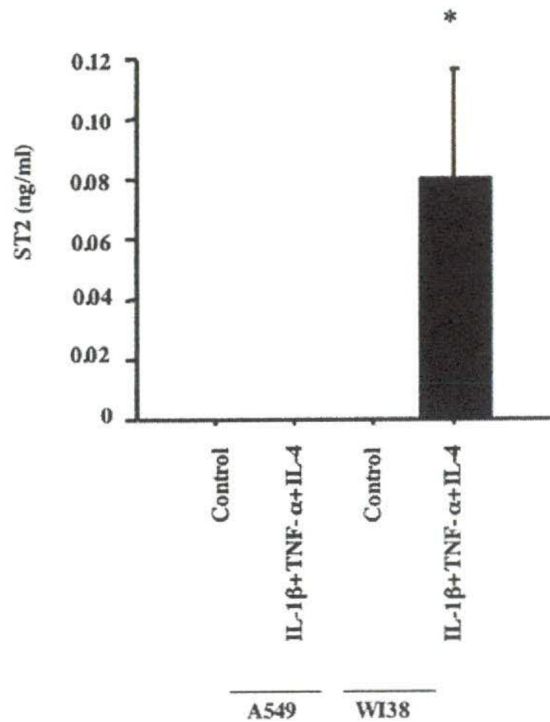

FIGURE 3 A combination of IL-1β, TNF-α, and IL-4 on ST2 production by A549 and WI38 cells. A549 and WI38 cells were stimulated with IL-1β (10 ng/mL) and TNF-α (100 ng/mL) plus IL-4 (100 ng/mL), respectively. After 36 hours, soluble ST2 protein in the supernatants was assayed by ELISA. The mean ST2 concentrations ± SEM are shown for the cumulative data from each of 3 samples in 3 independent experiments. $^*P < .05$ versus control production in the absence of cytokines (serum-free medium alone).

these cytokines for 36 hours, and we used RT-PCR to examine ST2 mRNA expression. The combination of IL-1β and IL-4 synergistically enhanced the expression of ST2 mRNA from A549 cells compared to the control level (Figure 2*A*). The amounts of ST2 mRNA expression by the combination of IL-1β and IL-4 did not differ from IL-1β plus TNF-α plus IL-4 in A549 cells. Therefore TNF-α might have minimal effect compared to IL-1β in A549 cells. The pattern of ST2 mRNA expression in WI38 cells was different from that in A549 cells: appreciable amounts of ST2 mRNA expression were generated by stimulation with IL-4 alone, by IL-1β plus IL-4, by TNF-α plus IL-4, and by IL-1β plus TNF-α plus IL-4 (Figure 2*B*). The ST2 mRNA expression by the combination of IL-1β plus TNF-α plus IL-4 tended to be enhanced compared to IL-1β plus IL-4, or TNF-α plus IL-4. These data demonstrated that the combination of IL-1β, TNF-α, and IL-4 had an additive effect on ST2 mRNA expression from WI38 cells. Furthermore, A549 and WI38 cells were stimulated with a combination of IL-1β, TNF-α, and IL-4 for 36 hours, and ST2 protein in the supernatants was quantified by specific ELISA. Importantly, costimulation with these 3 cytokines enhanced the ST2 protein level from WI38 cells (Figure 3). On the other hand, no detectable amounts of ST2 protein were released from A549 cells (Figure 3).

DISCUSSION

The results of the present study have demonstrated that ST2 gene expression is induced in the lung tissue of a BLM-induced lung fibrosis model. To our knowledge, this is the first report addressing the kinetics of soluble ST2 mRNA expression in murine models of BLM-induced pulmonary fibrosis. The kinetics of gene expression in the lung of the BLM-induced pulmonary fibrosis model demonstrated that the peak in gene expression of Th2-type cytokines (IL-4 and IL-5) and proinflammatory cytokines (IL-1β and TNF-α) occurred earlier than the peak in ST2 expression (Figure 1). Furthermore, the combination of IL-1β TNF-α, and IL-4 had an additive effect on ST2 mRNA expression in a human type II alveolar epithelial cell line, A549, and a human lung fibroblast cell line, WI38 (Figure 2). These findings suggest that the level of soluble ST2 genes may increase, possibly reflecting the development of the inflammatory process and the Th2-type immune response in the fibrotic lung tissue, and this may modulate the development of pulmonary fibrosis.

We have previously reported that the serum soluble ST2 protein levels are significantly elevated in patients with acute exacerbation of IPF, and that these levels correlate with disease severity [35]. To test whether the fibrotic process induces ST2 gene expression in vivo, we utilized a murine model of BLM-induced pulmonary fibrosis. In this model, ST2 and TGF-β1

mRNA expression of the lung increased to maximal levels at day 14 post BLM challenge. Furthermore, the kinetics of ST2 gene expression is statistically correlated with TGF-β1 mRNA expression and hydroxyproline levels (Table 3). These data suggest that ST2 might have a close relation to the fibrotic process in the lung of the BLM-induced pulmonary fibrosis model. Although the release of TGF-β from macrophages was not altered by soluble ST2 treatment [34, 41], the relationship between ST2 and TGF-β1 in fibroblast proliferation should be further analyzed.

The ST2 mRNA expression increased significantly between days 7 and 21 and then declined to the baseline levels after day 56 in BLM-treated lungs, but it is uncertain which cells, infiltrating cells, endogenous cells, or both, express ST2 mRNA in this model. ST2 gene products are expressed not only in Th2 cell but also in various lung structure cells. Kumar and colleagues showed that the mRNA of ST2 was expressed in mast cells, fibroblast, and vascular endothelial cells [33]. We also observed ST2 mRNA expression in human bronchial epithelial cells, type II pneumocytes, alveolar macrophages, pulmonary smooth muscle cells, pulmonary artery endothelial cells, and lung fibroblasts [34]. It is necessary to determine which cells express soluble ST2 mRNA in response to BLM exposure. Although immunohistochemical study of ST2 was done to determine which cells expressed soluble ST2 in the fibrotic lung, we could not detect ST2 protein by the histopathological study. Further analysis such as in situ hybridization will be needed to determine which cells express soluble ST2 in the fibrotic lung.

We investigated the capacity of alveolar epithelial cells and fibroblasts to induce ST2 mRNA in response to the combination of proinflammatory cytokine and Th2-type cytokine in vitro. The results of the present study have demonstrated that the combination of IL-1β, TNF-α, and IL-4 synergistically enhanced ST2 mRNA expression from A549 cells and WI38 cells (Figure 2). Furthermore, costimulation with these 3 cytokines enhanced the ST2 protein production from WI38 cells, but not from A549 cells (Figure 3). Although ST2 protein was detected in WI38 cells, the amount of ST2 protein was very low (approximately 0.08 ng/mL). We thought that ST2 protein from A549 cells is too low to detect by this ELISA kit (detection limit of ST2 protein is 0.032 ng/mL). To our knowledge, this is the first report certifying the synergistic effect of proinflammatory cytokine and Th2-type cytokine on ST2 expression in vitro. Recent data from our laboratory indicate that ST2 gene expression in Th2 cells is induced by the overexpression of the Th2-specific transcription factor GATA-3 via activation of the distal promoter region [42]. Further analyses are necessary to clarify the synergistic effect of proinflammatory cytokine and Th2-type cytokine on signal transducer and transcription factors involved in ST2/ST2L gene expression in various cells.

The biological function of the ST2 gene products remains unclear, but its homology with the Toll-like receptor (TLR) and other IL-1R members suggests that it may play a central role in innate and adaptive immune response. In murine asthma models, the administration of recombinant ST2 fusion protein or intravenous soluble ST2 gene transfer attenuates eosinophilic inflammation of the airway and suppresses IL-4 and IL-5 production, and these data suggest that soluble forms of ST2 protein can inhibit the interaction of ST2L (membrane-bound ST2) by competing with its putative ligand, resulting in down-regulation of Th2 effecter function [29, 43, 44]. As shown in Table 3, there was a strong correlation of ST2 mRNA with IL-5 mRNA expression in our experimental model. Oshikawa and colleagues have shown that there was a weak correlation of serum soluble ST2 protein levels with IL-5 in the patients with atopic asthma [31]. Taken together, these data suggest that ST2 is closely related with Th2-type cytokine. Further study to investigate ST2 expression in the Th2 and/or proinflammatory cytokine–blocked or gene-deficient animals should be needed. Furthermore, Sweet and colleagues showed that the administration of a recombinant ST2 fusion protein suppresses TNF-α production via down-regulation of TLR4 expression in macrophages and results in the significantly enhanced survival rate in murine lipopolysaccharides-induced shock models [41]. These findings suggest that soluble forms of ST2 protein may have a capability of inhibiting the production of proinflammatory cytokines and Th2-type cytokines. It seems unwise to extrapolate protective roles of ST2 gene products based on various experiments; however, we can speculate that increased endogenous ST2 gene expression in the lung may be required to suppress the fibrotic process, which is mediated by Th2-type cytokine and proinflammatory cytokine. In brief, ST2L promotes the development of Th2 response and suppresses the release of proinflammatory mediator [45, 46]. On the other hand, soluble ST2 acts as an inhibitor of ST2L signaling in Th2 response and as a direct suppressor in proinflammatory stimuli [46]. Experimental study utilizing intravenous ST2 gene transfer mice is underway to determine the role and function of ST2 gene products in the BLM-induced pulmonary fibrosis model.

In summary, here we demonstrated that ST2 gene expression was induced in the lung tissue of a murine model of BLM-induced pulmonary fibrosis in vivo. Furthermore, the combination of proinflammatory cytokine and Th2-type cytokine had an additive effect on ST2 expression in alveolar epithelial cells and fibroblasts in vitro. These findings suggested that up-regulated expression of the ST2 gene may modulate a process of pulmonary fibrosis. Further analysis will be needed to determine the role and function of the ST2 gene in pulmonary fibrosis.

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ORIGINAL ARTICLE

Preventive effect of hochu-ekki-to, a Japanese herbal medicine, on bleomycin-induced lung injury in mice

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Preventive effect of hochu-ekki-to, a Japanese herbal medicine, on bleomycin-induced lung injury in mice
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Objective: Pulmonary fibrosis is thought to be closely associated with the T-helper type-2 (Th2) immune response. Recent studies have shown that hochu-ekki-to (TJ-41), a Japanese herbal medicine, may correct the Th1/Th2 imbalance skewed to Th2. The present study was designed to investigate the preventive effect of TJ-41 on the development of bleomycin (BLM)-induced lung injury in mice.

Methods: Female C57BL/6 mice were divided into a group given ordinary feed and another group given the same feed plus TJ-41 mixed in at a dose of 1 g/kg/day. Both groups were maintained on this diet for 8 weeks before and 5 weeks after administration of 2 mg/kg BLM intratracheally.

Results: Mortality after BLM-induced lung injury was significantly lower in the TJ-41-treated mice. The hydroxyproline content and fluid content in the lung on day 35 was significantly lower in the TJ-41-treated mice. Histologically, TJ-41 reduced the number of infiltrating cells, thus ameliorating the destruction of the lung architecture, and attenuated the lung fibrosis score. Furthermore, TJ-41 inhibited the expression of the interleukin-5/interferon-γ mRNA ratio in the lung on day 7.

Conclusions: Treatment with TJ-41 partially prevented experimental lung fibrosis through the correction of the Th1/Th2 imbalance skewed to Th2.

Key words: bleomycin, hochu-ekki-to, kampo (traditional Japanese herbal) medicine, pulmonary fibrosis, T-helper type-2 cytokine.

INTRODUCTION

Interstitial pulmonary fibrosis is a consequence of many types of severe or sustained lung inflammation. Bleomycin (BLM), a mixture of glycopeptides derived from *Streptomyces verticillus*, is a potent chemotherapeutic agent and is known to produce pulmonary fibrosis in humans as well as in experimental animals. The mechanisms by which BLM causes pulmonary fibrosis are not yet clearly understood, but an initial lung injury may subsequently increase the influx of activated inflammatory cells into lung parenchyma. In pulmonary fibrosis, the inflammatory response is thought to be closely associated with a T-helper type-2 (Th2) immune response.[1] Interferon (IFN)-γ inhibits fibroblast proliferation and the production of collagen and other non-collagenous extracellular matrix proteins by the cell, while interleukin (IL)-4 promotes them.[2,3] *In vivo* studies using a BLM-induced pulmonary fibrosis model have supported this concept by showing that IL-4 mRNA expression is upregulated in lung fibrosis lesions and that IL-12 attenuates pulmonary fibrosis through the modulation of IFN-γ production.[4,5] These findings suggest

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Table 1 Crude drug composition of hochu-ekki-to

Crude drug	Composition (g)
Astragali radix	4.0
Atractylodis lanceae rhizoma	4.0
Ginseng radix	4.0
Angelicae radix	3.0
Bupleuri radix	2.0
Zizyphi fructus	2.0
Aurantii nobilis pericarpium	2.0
Glycyrrhizae radix	1.5
Cimicifugae rhizoma	1.0
Zingiberis rhizoma	0.5

that the persistent imbalance between T-helper type-1 (Th1) and Th2 cytokine expression in the lung may be an important underlying factor in the progression of lung injury and following pulmonary fibrosis.

Known in Japan as hochu-ekki-to (TJ-41) and elsewhere by its Chinese name, bu-zhong-yi-qi-tang, TJ-41 is composed of 10 species of medicinal plants and is used as a remedy for chronic diseases or weakness suffered after illness. It has previously been reported that the pre-administration of TJ-41 showed an inhibitory effect on lipopolysaccharide-induced acute lung injury in mice associated with the suppression of chemokine production.[6] Recent studies have shown that TJ-41 may correct the Th1/Th2 imbalance skewed to Th2. TJ-41's ability to correct the Th1/Th2 imbalance seems to underlie its effectiveness in the treatment of allergic dermatitis,[7] atopic dermatitis,[8,9] contact skin hypersensitivity[10] and allergic asthma.[11] TJ-41 is an oral IFN-γ inducer and consequently has an enhancing effect on Th1 responses during *Listeria monocytogenes* infection and a suppressive effect on Th2 responses, such as IgE production and eosinophil infiltration, in mice.[12] Ochi *et al.* have shown that TJ-41 suppresses porcine serum-induced liver fibrosis through the inhibition of IL-13 and transforming growth factor (TGF)-β1 production.[13] In the present study, the established model of BLM-induced lung injury in mice was used to investigate TJ-41's preventive effects on BLM-induced inflammation and fibrosis.

METHODS

Preparation of TJ-41

TJ-41 was authenticated and provided by Tsumura Co., Ltd. (Tokyo, Japan). The crude drug composition of TJ-41 is given in Table 1. The mixture of crude drugs was extracted with 600 mL of water at 100°C for 1 h. The decoction was filtered and then lyophilized to obtain a powdered extract. The yield of extract was 7.5 g. The dose of TJ-41 was adjusted to 1 g/kg body weight/day by mixing it with normal feed. Feed containing TJ-41 was produced by adding 0.5% (weight/weight) of powdered TJ-41 extract. The dose of TJ-41, 1 g/kg, was roughly equivalent to six to seven times the daily human dose (7.5 g/day). To evaluate the effect of TJ-41 administration on BLM-induced lung injury, mice given normal feed were compared with mice given the same feed mixed with TJ-41 for 8 weeks, followed by intratracheal BLM or saline injection in both groups.

BLM administration and treatment protocols

The study used C57BL/6 mice, a well-characterized inbred strain susceptible to BLM-induced lung injury. Specific, pathogen-free female C57BL/6 mice were obtained from Japan SLC, Inc. (Tochigi, Japan) and housed in an animal facility. All animal experiments were conducted with the permission of the institutional ethics committees for animal experiments. The C57BL/6 mice, 3 weeks of age, then consumed either normal feed or the feed mixed with TJ-41 for 8 weeks before the BLM challenge and until the end of the study. There were four study groups: the control group received normal feed for 8 weeks and saline injection; the TJ-41 group received the feed mixed with TJ-41 for 8 weeks and saline injection; the BLM group received normal feed for 8 weeks and BLM injection; and the TJ-41 + BLM group received the feed mixed with TJ-41 for 8 weeks and BLM injection. To induce lung injury and fibrosis, the mice were treated with intratracheal BLM (Nippon Kayaku, Tokyo, Japan) on day 0 (the mice were 11 weeks of age). The mice were anaesthetized with 0.01 mL/g of 10% pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL, USA) injected intraperitoneally, followed by intratracheal instillation of 2 mg of BLM/kg body weight in 50 μL of sterile isotonic saline. The control animals received intratracheal saline only. Blood was drawn from the atrium under anaesthesia on days 0, 7 and 35 after BLM instillation. After centrifugation at 3000 *g* for 10 min at 4°C, the serum level of potassium was determined by the ion-selective electrode method. BAL was carried out on day 7, and the left lung without BAL was removed and frozen to isolate RNA for RT-PCR. Histological examinations were carried out on day 0 (before BLM or saline injection) and on day 35. On day 35, the left lung was used for hydroxyproline assay.

BAL analysis

BAL was performed through a tracheal cannula with 0.7 mL of saline four times. Approximately 2.5 mL (90%) of BAL fluid (BALF) was recovered from each mouse examined. A 100-μL aliquot was used for the total cell count, and the remainder was immediately centrifuged at 1000 *g* for 10 min. The total cell count was obtained using a haemocytometer, and cell differentiation was determined on cytocentrifuge slides with Wright-Giemsa staining of more than 500 cells. The supernatants of BALF were stored at −80°C to measure the concentrations of IL-4 and IFN-γ, which were measured by a sandwich ELISA kit (Biosource International Inc., Camarillo, CA, USA) according to the manufacturer's instructions. Albumin

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concentration was determined in the cell-free BALF supernatant by dye-binding assay (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, CA, USA).

Morphological evaluation

Histopathological evaluation was carried out on the animals. We selected three mice randomly from each group. Both lungs were fixated by filling lungs through the tracheal cannula to 25 cm H_2O with 10% neutral buffered formaldehyde solution. The trachea was then occluded, and fixation was allowed to continue for 4–10 days before the study. Longitudinal tissue sections of the lung were embedded in paraffin, stained with haematoxylin–eosin, and examined by light microscopy at magnifications of ×40 and ×100. The pathological grades of inflammation and fibrosis in the whole area of the mid-sagittal section were evaluated under ×40 magnification. The pathological grade was determined according to the following criteria: (i) no lung abnormality; (ii) presence of inflammation and fibrosis involving less than 25% of the lung parenchyma; (iii) lesions involving 25–50% of the lung; and (iv) lesions involving more than 50% of the lung.[14] To reduce observer bias, three observers, two of whom were histopathologists, interpreted the images independently in a blinded fashion, and the mean of the three observers' findings was taken as the fibrotic score of the specimen.

Measurement of fluid content and assay of hydroxyproline

The wet lung weight of the left lobe was measured after careful excision of extraneous tissues. The lung was exposed for 48 h at 180°C and the dry weight was measured. Water content was calculated by subtraction of the dry weight from the wet weight. Hydroxyproline in the murine lung was assayed according to the commonly used procedure of colourimetric measurement (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo, Japan).[15] Briefly, lungs were harvested on each designated day after BLM administration and homogenized in 1 mL of PBS, pH 7.4, with a tissue tearer. One-half millilitre of each sample (left lung) was then digested in 1 mL of 6 N hydrochloric acid for 8 h at 120°C. Five microlitres of citrate–acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 mL of chloramine-T solution (282 mg of chloramine-T, 2 mL of n-propanol, 2 mL of H_2O and 16 mL of citrate–acetate buffer) were added to each 5 mL sample, and the samples were left at room temperature for 20 min. Next, 100 mL of Ehrlich's solution, 9.3 mL of n-propanol and 3.9 mL of 70% perchloric acid were added to each sample, and the samples were incubated for 15 min at 65°C. Samples were cooled for 10 min and read at 550 nm on a spectrophotometer. Hydroxyproline concentrations from 0 to 400 mg/mL were used to construct a standard curve. The hydroxyproline content of the left lung in each mouse is presented as μg/lung.

Table 2 Primer sequences

Gene	Primers	Product size
IFN-γ	F: CTGTTTCTGGCTGTTACTGC R: TCAGCAGCGACTCCTTTTCC	426 bp
IL-4	F: CATCCTGCTCTTCTTTCTCG R: GATGCTCTTTAGGCTTTCCAG	378 bp
IL-5	F: ACAAGCAATGAGACGATGAGG R: GCCTTCCATTGCCCACTCT	272 bp
TGF-β1	F: CGGACTACTATGCTAAAGAGG R: GTATTCCGTCTCCTTGGTTC	344 bp
β-actin	F: TGTCCCTGTATGCCTCTGGT R: ACTGTGTTGGCATAGAGGTC	477 bp

F, sense primer; R, antisense primer.

RT-PCR analysis of mRNA expression of IFN-γ, IL-4, IL-5 and TGF-β1

On day 7 after BLM instillation, the total RNA was extracted from the whole lung tissue of the left lung using Trizol reagent (Life Technologies, Inc., Rockville, MD, USA). Five-hundred nanograms of total RNA was reverse-transcribed and amplified using an RT-PCR kit (Takara, Tsukuba, Japan) under the following conditions: 94°C, 1 min; 57°C, 1 min; and 72°C, 1.5 min for each cycle as previously described.[16] The sequences of primers and the fragments of PCR products are shown in Table 2. The optimal numbers of PCR cycles were 38 for IFN-γ, IL-4, IL-5, TGF-β1 and β-actin. The amplified products were electrophoresed on 3.0% agarose gel, and the intensity of the DNA bands was quantified by densitometry (NIH Image 1.61) and normalized to the β-actin expression. The ratio of cytokine to β-actin for each mouse was then standardized to the mean for the control group.

Statistical analysis

Survival curves were estimated by the Kaplan–Meier method. Comparisons of all curves were carried out using the two-tailed log-rank test. Data were expressed as mean ± SEM. For multiple comparisons, one-way analysis of variance was used with Fisher's protected least-significant differences method as a post-hoc test. Differences between two variables were assessed with the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of TJ-41 on mortality in BLM-treated mice

The severe lung injury caused by BLM administration resulted in high mortality. The survival rate of the

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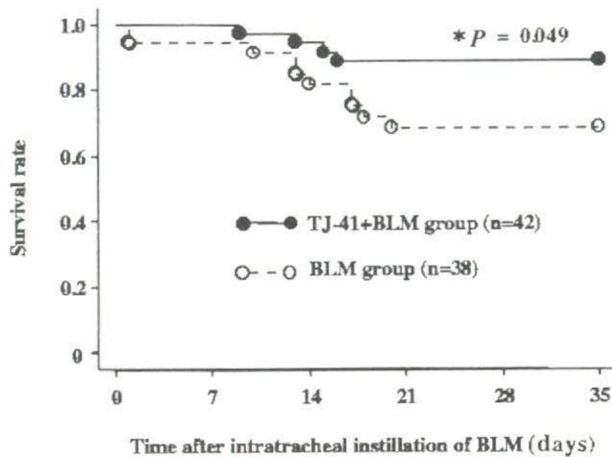

Figure 1 Effects of TJ-41 on mortality in bleomycin (BLM)-induced lung injury model. The group given an intratracheal instillation of BLM is shown by open circles (BLM group); closed circles show the TJ-41 + BLM group, which was given TJ-41 from 8 weeks before BLM instillation to the end of the experimental phase of the study. Overall survival rates for the TJ-41 + BLM group were significantly higher than those for the BLM group ($P = 0.049$). The control group ($n = 9$) and the TJ-41 group ($n = 8$) are not shown.

TJ-41 + BLM group was significantly higher than that of the BLM group ($P = 0.049$), using a two-tailed log-rank test (Fig. 1). The saline injection groups (control and TJ-41 groups) recorded no deaths.

Effects of TJ-41 on BALF cell analysis in BLM-treated mice

A total of 1 g/kg/day of TJ-41 was given for 8 weeks, and BLM was then instilled intratracheally. There was no significant difference in total cells, macrophages, neutrophils or lymphocytes in BALF in the BLM + TJ-41 group compared with the BLM group (data not shown). The albumin concentration in the BALF of the BLM-induced lung injury model was measured on day 7. However, pre-administration of TJ-41 did not improve the BLM instillation-induced elevation of albumin levels in BALF (data not shown).

Effects of TJ-41 on histological findings in lung tissue

Pre-administration of TJ-41 for 8 weeks revealed a normal alveolar structure without cellular infiltration or fibrous thickening (Fig. 2b). On day 35, after the BLM challenge, patchy fibrosis with destruction of the alveolar structure was observed mainly in the peribronchovascular regions in the BLM group (Fig. 2c). There were no striking differences between the histological pictures of the TJ-41 + BLM group and the BLM group (Fig. 2c,d). The microscopic findings were scored as described in the Materials and Methods. As shown in Figure 3, the pathological grade of inflammatory cell infiltration and pulmonary fibrosis at day 35 after the BLM challenge was significantly decreased by the administration of TJ-41 ($P = 0.0495$; BLM group vs TJ-41 + BLM group, 1.787 ± 0.257 vs 0.937 ± 0.219, respectively).

Effects of TJ-41 on fluid contents and hydroxyproline contents of the lung in BLM-treated mice

The injection of BLM elicited an inflammatory response characterized by the accumulation of water in the lung, as indicated by fluid content. Treatment with TJ-41 significantly reduced the fluid content on day 35 after BLM instillation (Fig. 4a). Figure 4b compares the hydroxyproline content of the lung between the four groups. No significant difference was observed between the control group (83.6 ± 2.1 μg/lung) and the TJ-41 group (86.3 ± 3.4 μg/lung). The subtle difference in the histological image associated with TJ-41 (Fig. 2c,d) may reflect a subtle difference in the hydroxyproline content. The hydroxyproline content of the lungs in the TJ-41 + BLM group (97.5 ± 3.7 μg/lung) was not significantly different from that in the TJ-41 group ($P = 0.195$), whereas it was significantly lower than in the BLM group (108.1 ± 4.0 μg/lung) ($P = 0.041$). These findings indicate that the administration of TJ-41 might be effective in preventing lung fibrosis induced by BLM.

Effects of TJ-41 on lung IFN-γ, IL-4, IL-5 and TGF-β1 mRNA expression in BLM-treated mice

Initially, the concentrations of IL-4 and IFN-γ in BALF were measured by ELISA, but neither were detected. Following this, RT-PCR was used to analyse IFN-γ, IL-4 and IL-5 mRNA expression in the lungs on day 7 after BLM exposure. The IFN-γ/β-actin mRNA ratio was slightly but not significantly upregulated in the TJ-41 + BLM group compared with the BLM group (Fig. 5b). No significant difference was observed in the IL-4/β-actin mRNA ratio among the four study groups (Fig. 5c). The IL-5/β-actin mRNA ratio in the BLM group was approximately 1.6-fold higher than the control group, a statistically significant difference (Fig. 5d). In the TJ-41 group, the lung IL-5/β-actin mRNA ratio was significantly reduced: approximately one-half that of the control group (Fig. 5d). Treatment with TJ-41 tended to reduce the increase in the IL-5/β-actin mRNA ratio by the administration of BLM (Fig. 5d).

To determine whether Th2 cytokine was upregulated compared with Th1 cytokine in lungs undergoing BLM-induced lung injury, the ratio of the Th2 to Th1 cytokine mRNA expression was analysed. The total lung IL-5/IFN-γ mRNA ratio in the BLM group was significantly (approximately 2.2-fold) higher than the control group (Fig. 6b). This elevation was significantly attenuated by the treatment with TJ-41 (Fig. 6b). On the other hand, no significant difference was observed in the IL-4/IFN-γ mRNA ratio among

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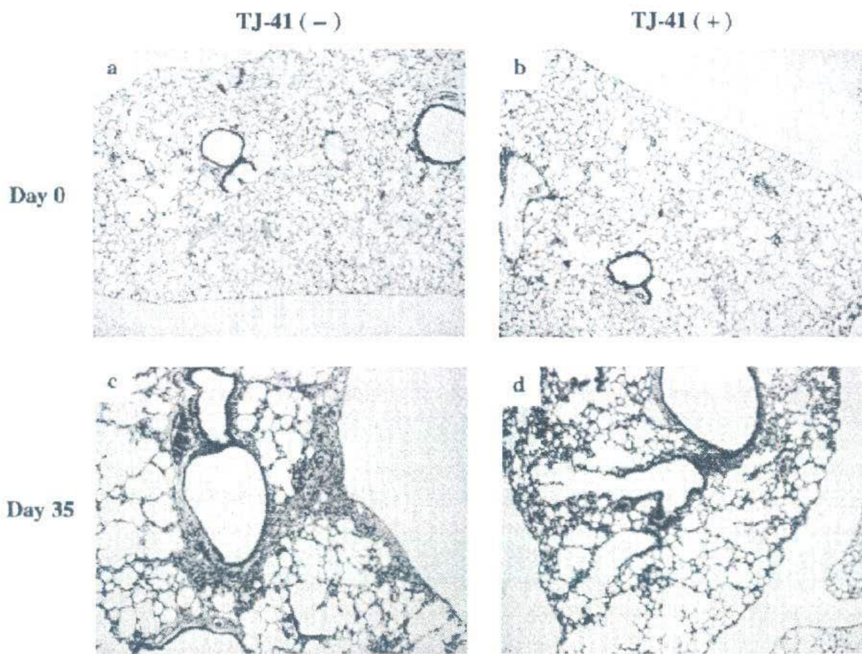

Figure 2 Effects of TJ-41 on histopathological changes in bleomycin (BLM)-induced lung injury model. Representative pictures of lung tissue specimens from mice receiving ordinary feed (a, c) and those receiving feed mixed with TJ-41 (b, d) (Original magnification, ×100).

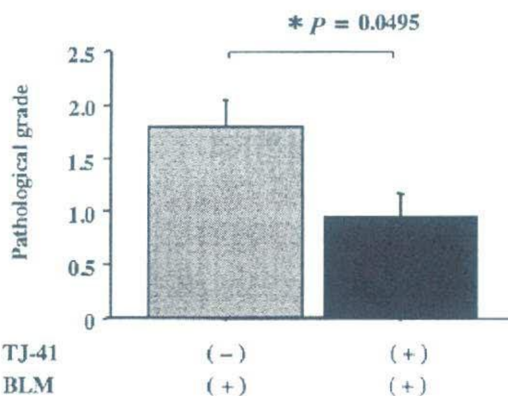

Figure 3 Effects of TJ-41 on lung histopathological score in bleomycin (BLM)-induced lung injury model. The lung histopathological score was compared between the two groups at day 35 after BLM challenge. The pathological grades of inflammatory cell infiltration and pulmonary fibrosis were significantly decreased by the administration of TJ-41 (black bar) compared with control (grey bar). Data are presented as means ± SEM ($n = 3$ per group). $^*P < 0.05$ in comparison with mice receiving ordinary feed after BLM challenge.

these four groups (Fig. 6a). The lung TGF-β1 mRNA expression by RT-PCR after the BLM challenge was investigated, but no difference in expression between the TJ-41 + BLM and BLM groups was found (data not shown).

DISCUSSION

The preventive effects of TJ-41 on a BLM-induced lung injury model were examined. Mortality was significantly lower in the TJ-41-treated mice. TJ-41 corrected the increase of the IL-5/IFN-γ mRNA ratio in the lung on day 7 after BLM administration. On day 35 after BLM administration, TJ-41 treatment significantly reduced the lung fluid content, lung hydroxyproline content and lung fibrosis score. The hydroxyproline contents reflected the histopathological changes of each group. These findings indicated that TJ-41 could prevent lung fibrosis in this experimental murine system, which in part may be associated with suppression of the Th2 cytokine.

The BLM-induced pulmonary fibrosis model constitutes a widely used model of human IPF that has significantly contributed to the current understanding of the disease. Although numerous agents targeting diverse signalling and molecular pathways have inhibited fibrosis very effectively in this model, none of these molecules has thus far demonstrated clear efficacy in the treatment of IPF. A main difference between human disease and the mouse model is the inflammatory component of early BLM-induced lung injury, which is often absent in human IPF.[17] Recently, Chaudhary *et al.* determined the time course of the development of inflammation and fibrosis in BLM-induced lung fibrosis.[18] Using an animal model with a single intratracheal injection of BLM, they showed that the 'switch' between inflammation and fibrosis occurred on or just after day 9.[18] Experimental therapeutic trials should include three arms with distinct periods of drug delivery: 0–35 days, 0–14 days (to dissect the impact on inflammation) and 14–35 days (to dissect the impact on fibrosis). In an ovalbumin-sensitized bronchial asthma mouse model, the oral administration of TJ-41 begun at the induction phase reduced both eosinophilia and Th2-type cytokine production in the airway, but TJ-41 given in the eliciting phase induced a predominant Th2 response with increased IgE production.[11] Nakada *et al.* stimulated

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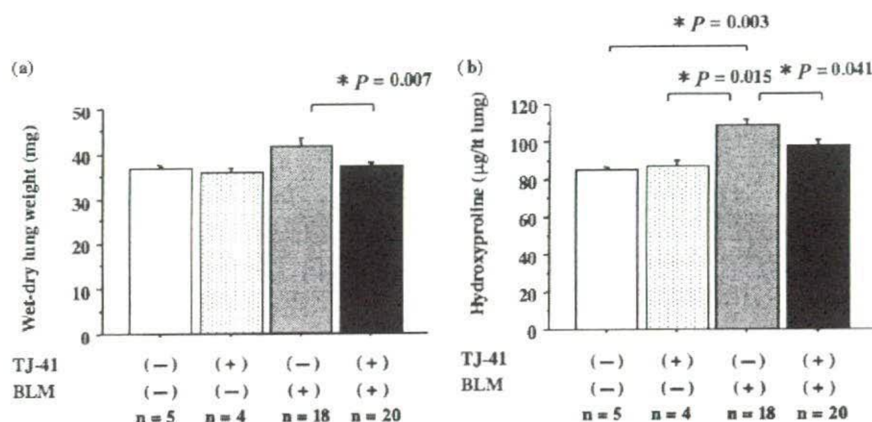

Figure 4 Effects of TJ-41 on fluid contents and hydroxyproline content of the lung in bleomycin (BLM)-treated mice. □, control group; □: TJ-41 group; ▨: BLM group; ■, TJ-41 + BLM group). (a) Effects of TJ-41 on the inflammatory response elicited by BLM injection and characterized by the accumulation of water in the lung as an indicator of oedema on day 35. Treatment with TJ-41 significantly reduced oedema formation induced by BLM. (b) Hydroxyproline content was significantly increased because of BLM injection. The administration of TJ-41 significantly attenuated the BLM-induced increase in hydroxyproline content on day 35. Data are presented as means ± SEM ($n = 5$ in control group, $n = 4$ in TJ-41 group, $n = 18$ in BLM group, and $n = 20$ in TJ-41 + BLM group).

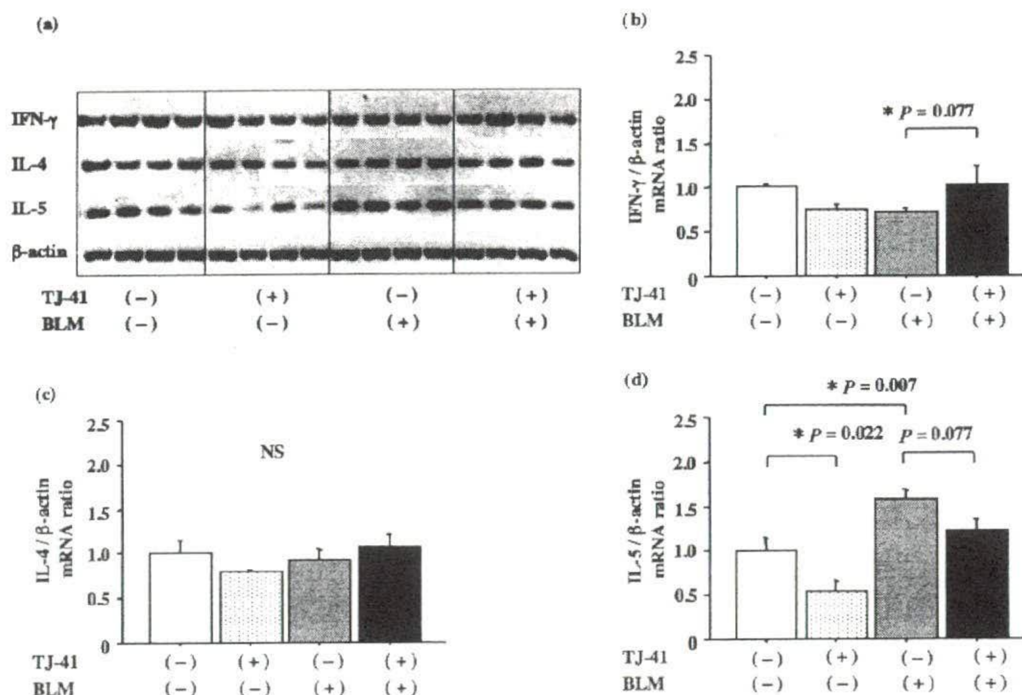

Figure 5 Effects of TJ-41 on IFN-γ, IL-4, and IL-5 mRNA expression of the lung tissue in bleomycin (BLM)-induced lung injury model. □, control group; ▤, TJ-41 group; ▨, BLM group; ■, TJ-41 + BLM group. (a) Total RNA was extracted from the lung at day 7 after intratracheal administration of BLM. The cDNA samples obtained by RT-PCR for IFN-γ, IL-4, IL-5 and β-actin were loaded on a 3.0% agarose gel. (b–d) Densitometric analyses of IFN-γ (b), IL-4 (c) and IL-5 (d) mRNA expression in the lung tissues were carried out. The relative levels designate the ratios obtained from the density of the bands of IFN-γ, IL-4 and IL-5 mRNA expression normalized to β-actin expression. The relative levels to the β-actin ratio of each mouse were standardized to the mean of that in the control group. The IL-5/β-actin mRNA ratio was significantly increased because of BLM injection (d). Data are presented as the mean relative densities ± SEM ($n = 4$ in each group in three independent experiments).

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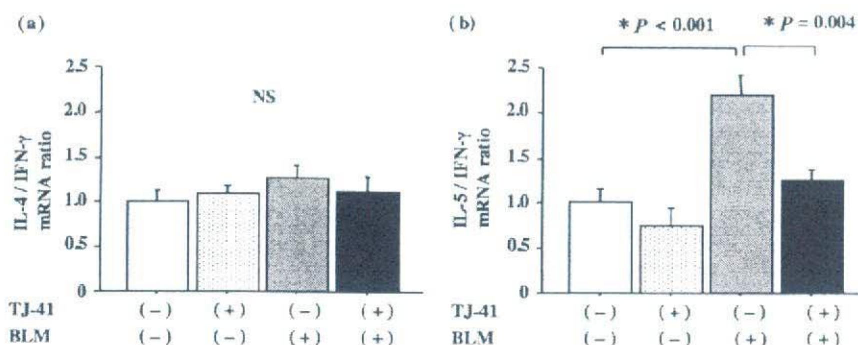

Figure 6 Effects of TJ-41 on Th1/Th2 cytokine imbalance of the lung in bleomycin (BLM)-induced lung injury model. □, control group; ▫, TJ-41 group; ▩, BLM group; ■, TJ-41 + BLM group. The data represent the ratios of the Th2/Th1-associated cytokine mRNA expression and are shown as the means ± SEM of the results from four animals at day 7 of the BLM challenge. Although the total lung IL-5/IFN-γ mRNA ratios in the BLM group were significantly increased compared with those of the control group, TJ-41 administration significantly attenuated this elevation (b).

spleen cells from TJ-41 administered mice for 2 or 4 weeks with soluble anti-CD3 mAb.[19] They found that although TJ-41 treatment for 2 weeks did not affect IL-4 concentration in the culture supernatants, TJ-41-treated mice for 4 weeks had lower IL-4 levels than control mice.[19] Thus, TJ-41 should be administered for at least 4 weeks to correct the Th1/Th2 imbalance. In the present study, TJ-41 was not expected to have a therapeutic effect on lung fibrosis, but to have a preventive effect in the very early stage of acute exacerbation in IPF.

IL-5, one of the Th2 cytokines, is a fibrogenic factor involved in the development of pulmonary fibrosis. Majumdar *et al.* have shown that the ratio of IL-5 to IFN-γ in IPF was significantly higher in the cases (22:1) than in the controls (4:1).[20] More importantly, the IL-5 mRNA expression was upregulated in mononuclear cells and eosinophils in areas of active fibrosis in BLM-treated mice,[21] and the anti-IL-5 antibody caused significant reductions in lung eosinophilia, TGF-β1 mRNA expression, monocyte chemotactic protein-1 mRNA expression and fibrosis in a murine BLM-induced pulmonary fibrosis model.[22] These results suggest the important role of IL-5 in pulmonary fibrosis. In the present study, although the changes in IL-5 and IFN-γ on their own are minimal (approximately 1.5-fold compared with controls) and reach statistical significance only by dividing IL-5 over IFN-γ (approximately 2.2-fold compared with controls), the results suggest that TJ-41's preventive effect against BLM-induced lung fibrosis might be associated with a reduction in IL-5 expression.

Gharaee-Kermani *et al.* have shown significant increases in lung IL-4 or IL-5 mRNA content between days 7 and 14 or between days 3 and 7, respectively, which decreased towards the control levels after day 21 of BLM-induced lung injury.[4,21] Keane *et al.* have shown that the levels of IFN-γ by specific ELISA in lung tissue were significantly lower on day 8 after BLM instillation than before instillation.[5] In the present study, the IFN-γ, IL-4 and IL-5 mRNA expression of the lung on day 7 after BLM instillation were investigated. Gao *et al.* have shown that TJ-41 tended to prevent not only the increase in IL-4 mRNA expression, but also the decrease in IFN-γ mRNA expression in the antigen-induced allergic dermatitis model in mice.[7] Furthermore, Tamura *et al.* showed that TJ-41 increased IL-18-induced IFN-γ production in human peripheral blood mononuclear cells.[23] The present study showed that the IL-4/IFN-γ mRNA ratio was not affected by TJ-41. The mouse strain used, C57BL/6, was different from that used in the previous reports.[4,5,21] Further analysis is needed to determine the effect of TJ-41 on various mouse strains.

There have been several reports on the therapeutic effects of herbal medicines on pulmonary fibrosis. Feitai, a composite formula consisting of several herbs, inhibited BLM-induced lung fibrotic lesions in a dose-dependent manner, as reflected by a decrease in lung hydroxyproline content and lung fibrosis fraction 28 days after BLM instillation in rats.[24,25] The effects *in vitro* on a WI-38 human lung fibroblast cell line showed that Feitai significantly reduced the cell proliferation and type I collagen synthesis stimulated by TGF-β.[25] Furthermore, Shen *et al.* have shown that Feitai treatment lessened BLM-induced lung fibrosis, at least in part, through the inhibition of TGF-β1 expression.[26] It was expected that TJ-41 would suppress TGF-β1 expression in a BLM-induced lung injury model. Although this study investigated lung TGF-β1 mRNA expression by RT-PCR after the BLM challenge, no difference was seen between the TJ-41 + BLM and BLM groups. TJ-41 consists of several herbs, as shown in Table 1. This study did not elucidate which herbal ingredient had the most suppressive effect on BLM-induced pulmonary fibrosis. Separate trials of some of the constituents have been already reported.[27] Considering these reports and the results of the present study, it is likely that the effect of each 'component' in TJ-41 would be too weak to prevent BLM-induced lung fibrosis. Therefore, it is proposed that the effects of combinations of two or more components in TJ-41 on the BLM-induced pulmonary fibrosis model be investigated.

Statistical analysis of the pathological grade of the sacrificed mice with or without TJ-41, at day 35 after

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