Suplatast tosilate prevents bleomycin-induced pulmonary fibrosis in mice

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Running title: SUPLATAST TOSILATE LIMITS LUNG FIBROSIS

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ABBREVIATIONS: AM, Alveolar macrophage; BAL, Bronchoalveolar lavage; BLM, Bleomycin; IPF, Idiopathic pulmonary fibrosis; LPS, Lipopolysaccharide; MCP-1,
Monocyte chemoattractant protein -1; NFκB, Nuclear factor κB; TGF-β, Transforming growth factor –β; DW: Distilled water; ST: Suplatast tosilate.

A recommended section is pulmonary section.
ABSTRACT

Increasing evidence suggests that the development of pulmonary fibrosis is a T helper 2 (Th2) -mediated process. Suplatastat tosilate is a Th2 cytokine inhibitor that is widely used as an asthma controller in Japan. Therefore, we hypothesized that suplatastat tosilate might have an inhibitory effect on the development of pulmonary fibrosis. To investigate this effect, suplatastat tosilate was administered to mice following the intratracheal instillation of bleomycin (BLM). The effect of suplatastat tosilate was studied by analysis of bronchoalveolar lavage (BAL) fluid and a hydroxyproline assay. We found that the treatment of mice with suplatastat tosilate significantly reduced the degree of pulmonary fibrosis. Because a significantly elevated Th2 response was not detected in the C57BL/6 mice following BLM administration, the effect of suplatastat tosilate on Th2 cytokines could not be demonstrated. Interestingly, however, the upregulation of the MCP-1 levels in the BAL fluid was found to be suppressed. Following this results, we also demonstrated that suplatastat tosilate effectively inhibited the production of MCP-1 in alveolar macrophages (AM). These findings suggest that suplatastat tosilate has both anti-inflammatory and anti-fibrotic effects which were associated with a suppressed MCP-1 expression in AMs. Suplatastat tosilate, which is already widely used in Japan, may thus warrant further consideration as a potentially
useful treatment for pulmonary fibrosis.
INTRODUCTION

IPF is defined as a specific form of chronic fibrotic interstitial pneumonia associated with the histopathological appearance of usual interstitial pneumonia. The median survival of patients with IPF is reported to be 3–4 yr from the onset of respiratory symptoms (2000). Despite such a poor prognosis, the etiology of IPF remains unknown, and no effective therapeutic strategy has yet been established. The effects of current immunosuppressive therapy with corticosteroids and cytotoxic agents are limited, and the adverse effects cannot be ignored. Therefore, the establishment of an alternative therapeutic strategy is urgently needed.

Since the overall cytokine pattern in biopsies and alveolar macrophages from patients with IPF appears to be more Th2-type (i.e., IL-4, IL-5, and IL-13) than Th1-type (i.e., IL-12 and IFN-γ) (Wallace et al., 1995; Furuie et al., 1997), a biased Th2 cytokine profile in the lungs is regarded as one of the causes of IPF and, thus, a therapeutic strategy to correct this bias seems to be a promising approach. To the best of our knowledge, INF-γ, a major Th1 cytokine, is the only agent used for IPF patients in clinical studies as a therapeutic strategy targeted inhibition of Th2 cytokines. The rationale for its use was also based on its properties as an inhibitor of fibroblast proliferation, collagen synthesis and deposition. A recent meta analysis study showed
INF-γ therapy to be associated with a reduced mortality (Bajwa et al., 2005), however, a randomized controlled trial involving 330 patients failed to show any clear benefit from INF-γ therapy in comparison to a placebo (Raghu et al., 2004; King et al., 2005).

Seeking other means to modulate Th2 cytokines in the lungs stimulated interest in suplatast tosilate (IPD-1151T, Fig. 1), which is widely used as an asthma control drug in Japan, because it has been shown to have an inhibitory effect on the production of Th2 cytokines (IL-4, IL-5, and IL-13) in animal models of bronchial asthma (Iijima et al., 1999; Zhao et al., 2000; Matsumoto et al., 2002). Previous pharmacokinetics studies have shown that, after a single oral administration, suplatast tosilate is readily distributed into blood cells, while staying in the cells with a half-life of 12 days, and an active bioavailability of suplatast tosilate in the lung tissue has also been confirmed (Kuwata T, et al., 1992). Based on these findings, it is possible that oral administration of suplatast tosilate may limit the development of pulmonary fibrosis through the inhibition of Th2 cytokines. To test this concept, the effects of suplatast tosilate on bleomycin-induced pulmonary fibrosis in C57BL/6 mice were investigated. The degree of pulmonary fibrosis was assessed by measuring the hydroxyproline content in the lungs, and the concentrations of Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-13, in bronchoalveolar lavage (BAL) fluid were measured. In addition, the levels of MCP-1, a
chemokine associated with Th2 polarization (Karpus et al., 1997; Gu et al., 2000; Matsukawa et al., 2000), and TGF-β, a cytokine strongly involved in the fibrogenic process, in BAL fluid were also assessed.
METHODS

Animals

Specific pathogen-free, female C57BL/6 mice weighing 17 to 20 g were purchased from CLEA Japan (Tokyo, Japan). The animals were housed in pathogen-free rooms and maintained on laboratory chow, with free access to food and water. The committee on animal research at the University of Hiroshima approved all procedures.

Bleomycin exposure

The mice were anesthetized with intraperitoneal pentobarbital, and the trachea was exposed through a cervical incision. Bleomycin (BLM, 1.5 mg/kg body weight; Nippon Kayaku Co., Tokyo, Japan) was dissolved in phosphate buffered saline (PBS) and then instilled intratracheally with a 27-gauge needle.

Administration of suplatast tosilate

Suplatast tosilate (IPD–1151T: (6)-[2-[4-(3-ethoxy-2-hydroxypropoxy)phenylcarbamoyl] ethyl] dimethylsulfonium p-toluenesulfonate, Fig. 1) was synthesized and supplied by the Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). The suplatast tosilate powder was dissolved in distilled water and administered into mice by oral gavage.
Experimental protocol

After BLM was intratracheally administered, the mice were randomly divided into two groups, namely, BLM + suplatast tosilate (ST) group and BLM + Distilled water (DW) group. The mice of the BLM + ST group were given suplatast tosilate (100 mg/kg/day) by oral gavage daily until sacrificed; the mice of BLM + DW group received an equivalent volume of water. The control group received an equivalent volume of water after administrated PBS intratracheally. Animals were sacrificed 7, 14, and 21 days after BLM administration. Each group had 8 mice at each time point. The control group (n = 8 mice) was sacrificed at 21 days after intratracheal instillation of PBS.

Bronchoalveolar lavage (BAL) and cell analysis

The mice were sacrificed with a lethal dose of pentobarbital, the trachea was cannulated with an 18-gauge needle, and the lungs were lavaged twice with 1 ml of PBS. The lavage fluids were pooled and were centrifuged at 300 g for 10 min at 4°C. The supernatants were stored at -80°C for the measurement of cytokine concentrations. The cell pellets were resuspended in 1 ml of Dulbecco’s modified Eagle’s medium, and the
total cell numbers were counted with a hemocytometer. Differential cell counts were
determined by counting at least 300 cells on a smear prepared using cytospin (Shandon
Inc., Pittsburgh, Pennsylvania, USA) and stained with Diff-Quick (Kokusai Shiyaku,
Kobe, Japan).

**Histopathology**

The lung tissue specimens of the mice were fixed by inflation with a buffered 10% formalin solution. Lung tissue specimens were embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E) and thereafter were examined by light microscopy.

**Cytokine measurements**

Commercially available ELISA kits for INF-γ, IL-4, IL-5, IL-6, IL-13, total and active TGF-β, and MCP-1 were obtained from R&D Systems (Minneapolis, MN). The concentration of each cytokine in the sample was measured following the manufacturer’s protocols.

**Hydroxyproline assay**
To assess lung fibrosis biochemically, lung tissues were homogenized and then analysed for hydroxyproline content after acid hydrolysis, as described previously (Woessner, 1961). Briefly, lung tissues were hydrolysed with 12N hydrochloric acid at 110°C for 24 hr. After neutralization with sodium hydroxide, the hydrolysates were diluted with distilled water. Hydroxyproline in the hydrolysates was assessed colourmetrically at 550 nm for p-dimethylaminobenzaldehyde. The results were expressed as micrograms of hydroxyproline contained in total lung tissue.

**Preparation of AMs**

AMs were isolated as previously described with slight modifications (Kaltreider et al., 1988). Briefly, the mice were sacrificed with a lethal dose of pentobarbital and the trachea was cannulated with an 18-gauge needle. The lungs were lavaged 5 times with 1 ml of PBS containing 0.1% EDTA. The lavage fluids were pooled and centrifuged at 300 g for 10 min at 4°C. The cell pellet was washed once with PBS and resuspended in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. The cells were plated at a cell density of $3 \times 10^5$ cells per well in 96-well plates, incubated for 45 min at 37°C in 5% CO₂, and gently washed three times with RPMI 1640 medium containing 10% FCS to remove unattached cells. Greater than 95% of the
plastic-adherent cells obtained from normal mice and around 90% of the plastic-adherent cells obtained from BLM-injured mice were morphologically macrophages.

Assessment of MCP-1 production in AMs

First, the cells adhering to the plastic were isolated from the mice intratracheally instilled with BLM 7 days before and, then, incubated in the presence or absence of suplatast tosilate at concentrations of 10 and 100 µg/ml. Twenty four hours after the start of incubation with suplatast tosilate, the culture supernatants were collected and subjected for MCP-1 measurement. Next, the cells adhering to the plastic were isolated from normal mice. Following preincubation with or without suplatast tosilate for 3 hr, these cells were then incubated with LPS at the concentration of 1 µg/ml in the presence or the absence of suplatast tosilate. Twenty four hr after the addition of LPS, the culture supernatants were collected and MCP-1 concentration was measured.

Statistics

The results are expressed as the mean ± SEM. Differences between treatment groups were analyzed using ANOVA with Fisher’s PLSD test for pairwise comparisons.
(StatView; Abacus Concepts Inc., Berkeley, California, USA). A p value of less than 0.05 was considered statistically significant.
RESULTS

Effect of suplatast tosilate on BLM-induced pulmonary fibrosis

To determine whether suplatast tosilate has an anti-fibrotic effect on the development of pulmonary fibrosis, mice were treated with suplatast tosilate after BLM was intratracheally instilled. Fourteen and 21 days after BLM administration, the lungs were excised and collagen accumulation in the lungs was evaluated by measuring hydroxyproline content in the lungs. As shown in Fig. 2A, treatment with suplatast tosilate significantly suppressed the increase in the collagen content in the lungs of the BLM administered mice. To assess the histological changes, the lungs were excised and the sections were stained with H&E at 21 days after BLM administration. The lesions with inflammation and fibrosis in the lungs of the BLM administered mice treated with suplatast tosilate (Fig. 2C) significantly decreased in comparison to those in the lungs of the BLM administered mice without suplatast tosilate treatment (Fig. 2B).

Effect of suplatast tosilate on BLM induced lung inflammation

To evaluate an effect of suplatast tosilate on the inflammatory responses induced by BLM, inflammatory cells recovered in the BAL fluids were analyzed on 7, 14, and 21 days after intratracheal administration of BLM. The recovery rate of BAL fluid typically
exceeded 85% and did not differ between the experimental groups. As shown in Fig. 3, the numbers of total inflammatory cells, macrophages, lymphocytes, and neutrophils in the BAL fluid were significantly elevated in the BLM injected mice compared with the PBS injected mice. Treatment with suplatast tosilate significantly decreased the number of total inflammatory cells, lymphocytes, and macrophages in the BAL fluid on day 14, and the number of neutrophils in the BAL fluid 7 and 14 days after BLM administration (Fig. 3).

Effect of suplatast tosilate on the cytokine levels in BAL fluid from BLM injected mice

To further analyze the effect of suplatast tosilate on the inflammatory responses induced by BLM, cytokine levels in the BAL fluid were measured on 7, 14, and 21 days after the intratracheal administration of BLM. The concentrations of Th1 (INF-γ) and Th2 (IL-4, IL-5, IL-6, and IL-13) cytokines were first measured in the BAL fluid. The levels of INF-γ and IL-4 in the BAL fluids were found to be nearly equal to or lower than the minimal detectable limits for each ELISA system (data not shown). As a result, we omitted these two cytokines from the analysis. In comparison to the control mice, the levels of IL-5 and IL-13 in BAL fluid were not significantly elevated in
BLM-injected mice except for IL-13 level on day 21, and treatment with suplatast tosilate did not change these levels (Figs. 4A and C). In contrast, the intratracheal administration of BLM induced significant increases of IL-6 in BAL fluid, and treatment with suplatast tosilate significantly reduced the IL-6 level in BAL fluid 7 days after BLM administration (Fig. 4B). Thereafter, the levels of MCP-1, a chemokine associated with Th2 polarization, and total and active TGF-β, a cytokine strongly involved in fibrogenic process, were measured in the BAL fluid. The MCP-1 levels in the BAL fluid were significantly elevated in the BLM-injected mice in comparison to the PBS injected mice 7 and 14 days after BLM administration, and, interestingly, treatment with suplatast tosilate was found to dramatically reduce these levels (Fig. 5A). The levels of total TGF-β in BAL fluid also significantly increased in the BLM-injected mice compared with PBS injected mice on 7, 14, and 21 days after BLM administration, however, treatment with suplatast tosilate did not decrease these levels in a statistically significant manner (Fig. 5B). Although their magnitudes were almost negligible in comparison to those of total TGF-β, the levels of active TGF-β in the BAL fluid were also significantly elevated in the BLM-injected mice in comparison to the PBS injected mice at 7, 14, and 21 days after BLM administration. Interestingly, treatment with suplatast tosilate significantly reduced these levels at 14 and 21 days after BLM
administration (Fig. 5C).

**Effect of suplatast tosilate on MCP-1 production by alveolar macrophages (AMs)**

MCP-1 has been found to be expressed in various type of cells including fibroblasts, endothelial cells, and mast cells, however, the major source of MCP-1 in the lungs of BLM-injected mice seems to be macrophages (Shen et al., 2005). Based on strong reduction of the MCP-1 level in the BAL fluid from BLM-injected mice treated with suplatast tosilate, it was possible that the suplatast tosilate had a direct effect on the MCP-1 production from AMs. We first evaluated the effect of suplatast tosilate on MCP-1 production from AMs isolated from BLM-injected mice (BLM-stimulated AMs). BLM-stimulated AMs in BAL fluid were collected 7 days after BLM administration and incubated with or without suplatast tosilate. As shown in Fig. 6A, the expression of MCP-1 in BLM-stimulated AMs decreased dose-dependently in the presence of suplatast tosilate. Thereafter, the effect of suplatast tosilate on MCP-1 production from naive AMs stimulated with inflammatory mediators was evaluated. Lipopolysaccharide (LPS) was chosen as an inflammatory mediator because a previous study demonstrated LPS to be the strongest inducer of MCP-1 expression in AMs (Brieland et al., 1995). Resident AMs in BAL fluid were collected from normal C57BL/6 mice and then were
incubated with LPS in the presence or absence of suplatast tosilate. As shown in Fig. 6B, suplatast tosilate showed an inhibitory effect on the upregulated production of MCP-1 from naive AMs stimulated with LPS.
DISCUSSION

Suplatast tosilate is a novel antiallergic agent which is only available in Japan. This agent has been shown to possess an inhibitory effect on the production of Th2 cytokines (IL-4, IL-5, and IL-13) in animal models of bronchial asthma (Iijima et al., 1999; Zhao et al., 2000; Matsumoto et al., 2002). Based on these data, it is possible that suplatast tosilate might have an antifibrotic effect on an animal model of BLM-induced pulmonary fibrosis through the inhibition of Th2 cytokines. In the present study, the antifibrotic effect of suplatast tosilate was demonstrated by the reduced hydroxyproline content in the lungs of BLM-injured mice treated with suplatast tosilate. Contrary to our hypothesis, however, an analysis of BAL fluids revealed that suplatast tosilate did not affect the concentrations of Th2 cytokines but significantly reduced MCP-1 levels. In addition, suplatast tosilate reduced the production of MCP-1 in AMs. Furthermore, suplatast tosilate was found to decrease the levels of IL-6 in BAL fluids from BLM-injured mice.

A most interesting finding of the current study is that the treatment of BLM-injured mice with suplatast tosilate was found to effectively suppress the increased MCP-1 levels in BAL fluids. In addition, suplatast tosilate also was able to reduce the production of MCP-1 in AMs. Although MCP-1, which is produced by various types of
cells (Rollins, 1996; Boring et al., 1998), is an important chemoattractant for mononuclear cells, a relationship has also been clearly demonstrated between MCP-1 and the development of pulmonary fibrosis (Zhang et al., 1994). The MCP-1 levels in the sera and BAL fluids from IPF patients were also shown to be significantly higher than those in healthy subjects (Car et al., 1994; Suga et al., 1999). MCP-1 mRNA and protein are strongly expressed in epithelial cells, macrophages, and endothelial cells in the lungs of IPF patients (Antoniades et al., 1992; Iyonaga et al., 1994). In BLM-induced pulmonary fibrosis in rats, MCP-1 mRNA expression in lung tissue was significantly elevated between days 3 and 21 (Zhang et al., 1994). Moore et al. reported that CC chemokine receptor 2 (CCR2; receptor of MCP-1, MCP-3, and MCP-5 in mice)-deficient mice were protected from FITC- and BLM-induced pulmonary fibrosis (Moore et al., 2001). Furthermore, anti-MCP-1 gene therapy blocking its signal transduction through CCR2 was shown to attenuate the development of BLM-induced pulmonary fibrosis. In addition to its chemoattractant activity, MCP-1 is believed to possess direct profibrogenic effects because of its ability to stimulate fibroblast collagen expression (Gharae-Kermani et al., 1996). Based on the observed close relationship between MCP-1 and pulmonary fibrosis, the antifibrotic effect of suplatast tosilate in BLM-induced pulmonary fibrosis can thus be considered to result from the suppression
of MCP-1 expression in the lung. The expression of MCP-1 has been shown to be elevated on day 3 after BLM administration while it persisted until day 21 (Zhang et al., 1994). In the present study, the MCP-1 levels in BAL fluid on day 7 after BLM administration dramatically increased and were still found to be significantly elevated on day 21 in the BLM-injured mice. Considering the expression pattern of MCP-1 in an animal model of BLM-induced pulmonary fibrosis, we believe that the treatment of the BLM-injured mice with suplatast tosilate, which started on the next day that BLM administration was begun and then continued daily until scarified, was necessary to elucidate the antifibrotic effect of suplatast tosilate mediated by the suppression of MCP-1.

In addition to MCP-1, the treatment of BLM-injured mice with suplatast tosilate significantly reduced the concentration of IL-6 in the BAL fluid 7 days after BLM administration. IL-6 is released by a variety of cells, including fibroblasts, and it has also been shown to mediate many inflammatory processes in the lung (Taga, 1997). In animal models, the lung-specific overexpression of IL-6 has been shown to produce lymphocytic alveolitis but little fibrosis (Denis, 1992; DiCosmo et al., 1994; Yoshida et al., 1995). Although in vitro studies have demonstrated the involvement of IL-6 in fibroblast proliferation, its role in fibrosis is still largely unknown (Knight et al., 2003).
These previous reports suggest that IL-6 is an important pro-inflammatory cytokine to mediate the inflammatory processes in the lung, and the results of the present study demonstrated that suplatast tosilate has an anti-inflammatory effect through the suppression of IL-6.

Contrary to our original hypothesis, this study did not show suplatast tosilate to have an inhibitory effect on the production of Th2 cytokines in the model of BLM-induced pulmonary fibrosis using C57BL/6 mice. An analysis using commercially available ELISA kits revealed that the concentrations of IL-5 and IL-13 but not IL-4 in the BAL fluids from BLM-administered mice to be measurable. However, the levels of these cytokines were not significantly elevated in comparison to those of normal mice, thus indicating that the changes of Th2 cytokines in BAL fluids from BLM-injured C57BL/6 mice are very limited. In addition, suplatast tosilate did not affect the levels of Th2 cytokines in BAL fluids from BLM-administered C57BL/6 mice, in contrast to its successful suppression of Th2 cytokines in BAL fluids from a bronchial asthma model of Balb/C mice (Zhao et al., 2000). These data suggest that suplatast tosilate does not have an inhibitory effect on Th2 cytokines in BLM-induced pulmonary fibrosis. However, we believe that this conclusion is premature because the changes in the levels of these Th2 cytokines are too small in the BAL fluids obtained from BLM-injured
mice.

Previous studies demonstrate that suplatast tosilate inhibits the release of the products of Th2 cells. Despite of an extensive search for the molecular target of suplatast tosilate, however, the precise mechanism of the effect of suplatast tosilate is largely unknown. In the present study, the potential of suplatast tosilate to reduce the production of MCP-1 and IL-6 in the lung was demonstrated. Both MCP-1 and IL-6 are induced by the activation of NFκB. This suggests that the NFκB pathway may be a target of suplatast tosilate. However, further investigation is needed.

In conclusion, this study found that suplatast tosilate treatment limited the development of pulmonary fibrosis induced by BLM. Contrary to the original hypothesis, suplatast tosilate did not decrease the levels of Th2 cytokines in BAL fluids. However, suplatast tosilate significantly reduced the MCP-1 level in the BALF from BLM-treated mice. Suplatast tosilate was also found to reduce the production of MCP-1 in AMs. Considering that MCP-1 is a pro-inflammatory chemokine, which is strongly involved in the development of pulmonary fibrosis, these results suggest that suplatast tosilate has both an anti-inflammatory and anti-fibrotic effect through the suppression of MCP-1 production in BLM-induced pulmonary fibrosis. In addition, the presence of constantly overexpressed MCP-1 in the lungs of IPF patients imply the possibility that
suplatast tosilate could also potentially be used for the treatment of pulmonary fibrosis.
REFERENCES


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FOOTNOTES


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LEGENDS FOR FIGURES

Figure 1. Chemical structure of suplatast tosilate (IPD–1151T)

Figure 2. Effect of suplatast tosilate on bleomycin-induced pulmonary fibrosis.

Following the intratracheal instillation of bleomycin (1.5 mg/kg), suplatast tosilate (100 mg/kg/day) or distilled water was administered daily to mice by oral gavage. (A) Fourteen and 21 days after the bleomycin administration, the lungs were excised and hydroxyproline content in the lungs were measured. The increased amount of hydroxyproline after BLM induction was inhibited by suplatast tosilate. Data are shown as the mean ±SEM for 8 mice per group at each time point. (*p<0.05 versus control group; †p<0.05 versus BLM + DW group). The lungs harvested at 21 days after the bleomycin administration were subjected to H&E staining. One representative example out of four or five is shown for (B) BLM + DW group and (C) BLM + ST group. Internal scale bars=50 μm


Figure 3. Effect of suplatast tosilate on the cell analysis of BAL fluid in bleomycin-injured mice.
Following the intratracheal instillation of bleomycin (1.5 mg/kg), suplatast tosilate (100 mg/kg/day) or distilled water was administered daily to mice by oral gavage. BAL fluid specimens were obtained 7, 14, and 21 days after the bleomycin administration, and total cell counts and differential cell counts were performed. Data are shown as the mean ±SEM for 8 mice per group at each time point. (*p<0.05 versus control group; †p<0.05 versus BLM + DW group).


Figure 4. Effect of suplatast tosilate on Th-2 cytokine levels in the BAL fluid from bleomycin-injured mice.

Following the intratracheal instillation of bleomycin (1.5 mg/kg), suplatast tosilate (100 mg/kg/day) or distilled water was administered daily to mice by oral gavage. BAL fluids were obtained 7, 14, and 21 days after the bleomycin administration. The concentrations of the cytokines in the BAL fluids were measured using commercially available ELISA kits. Note that IL-4 levels in the BAL fluid were nearly equal to or under the detectable limits of the ELISA kit, thus the data for IL-4 were not shown. Data are shown as the mean ±SEM for 8 mice per group at each time point. (*p<0.05 versus control group; †p<0.05 versus BLM + DW group).

**Figure 5. Effect of suplatast tosilate on levels of MCP-1 and TGF-β in the BAL fluid from bleomycin-injured mice.**

Following the intratracheal instillation of bleomycin (1.5 mg/kg), suplatast tosilate (100 mg/kg/day) or distilled water was administered daily to mice by oral gavage. BAL fluid specimens were obtained 7, 14, and 21 days after the bleomycin administration. The concentrations of (A) MCP-1, (B) total and (C) active TGF-β in the BAL fluids were measured using commercially available ELISA kits. Data are shown as the mean ±SEM for 8 mice per group at each time point. (*p<0.05 versus control group; †p<0.05 versus BLM + DW group).


**Figure 6. Effect of suplatast tosilate on the production of MCP-1 in AMs**

(A) MCP-1 production in the AMs isolated from the mice intratracheally instilled with bleomycin. AMs were prepared as described in the Materials and Methods, and, then, incubated in the presence or absence of suplatast tosilate at concentrations of 10 and 100µg/ml. Twenty four hours after, the culture supernatants were collected and assayed.
for MCP-1. (B) MCP-1 production in the LPS- stimulated AMs isolated from normal mice. AMs were prepared as described in Materials and Methods. Following preincubation with or without suplatast tosilate for 3 h, these cells were then incubated with LPS at the concentration of 1 µg/ml in the presence or absence of suplatast tosilate. Twenty four hours after the addition of LPS, the culture supernatants were collected and assayed for MCP-1.

ST: suplatast tosilate, LPS: lipopolysaccharide.
Figure 1
Figure 2

(A)

Hydroxyproline content (µg/ml)

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<th>14 days</th>
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(B) [Image of tissue section]

(C) [Image of tissue section]
Figure 3

(A) Total cells

(B) Macrophage

(C) Lymphocyte

(D) Neutrophil

Cell x10^4/ml

Cell x10^3/ml

BLM + DW + ST

7days 14days 21days

Control

* †

BLM + DW + ST

7days 14days 21days

Control

* †

BLM + DW + ST

7days 14days 21days

Control

* †

BLM + DW + ST

7days 14days 21days

Control

* †

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Figure 4

(A) IL-5 (pg/ml)

(B) IL-6 (pg/ml)

(C) IL-13 (pg/ml)
Figure 5

(A) MCP-1 (pg/ml)

(B) Total TGF-β (pg/ml)

(C) Active TGF-β (pg/ml)
Figure 6

(A) MCP-1 (pg/ml)

- ST (μg/ml)
  - (-) 10 100
  - (+) 10 100

(B) MCP-1 (pg/ml)

- LPS (1μg/ml)
  - (-) (+) (+) (+)

- ST (μg/ml)
  - (-) 10 100

p<0.001
p<0.005
p<0.005
p<0.001
p<0.05