

Pranlukast, a Cysteinyl Leukotriene Type 1 Receptor Antagonist, Attenuates the Progression but Not the Onset of Silica-Induced Pulmonary Fibrosis in Mice

Chiko Shimbori Naotaka Shiota Hideki Okunishi

Department of Pharmacology, Shimane University School of Medicine, Shimane, Japan

Key Words

Cysteinyl leukotrienes · Cysteinyl leukotriene type 1 receptor · Pulmonary fibrosis

Abstract

Background: Although cysteinyl leukotrienes (CysLTs) have been implicated in the etiology of acute inflammatory diseases, recent studies have suggested that they also directly stimulate fibroblasts. However, their precise role in the pathogenesis of pulmonary fibrosis is unclear. **Methods:** In this study, we evaluated the effect of both short- and long-term treatment with pranlukast, a CysLT type 1 (CysLT₁) receptor antagonist, on silica-induced pulmonary fibrosis in mice, which is characterized by persistent progression of fibrosis in the chronic phase. Pranlukast (30 mg/kg/day) was administered orally to mice for 2 or 10 weeks after intratracheal silica instillation. **Results:** Pranlukast treatment for 10 weeks significantly attenuated the progression of pulmonary fibrosis, and decreased the content of CysLTs and LTB₄, which were markedly increased in the bronchoalveolar lavage fluid (BALF) and lung tissues of silica-instilled mice in the chronic phase. However, pranlukast treatment for 2 weeks neither affected the acute inflammatory response induced by silica instillation nor inhibited the onset of fibrosis. The expression of TGF- β 1 and TNF- α was not affected by pranlukast treatment for either 2 or 10 weeks. **Conclusions:**

Pranlukast attenuates the progression of pulmonary fibrosis in the chronic phase but has no effect on the acute inflammatory response or on the onset of pulmonary fibrosis. The antifibrotic effect of pranlukast may be exhibited by antagonizing the direct profibrotic effect of CysLTs, without affecting the expression of other profibrotic cytokines such as TGF- β 1 and TNF- α , and also by decreasing the production of CysLTs and LTB₄.

Copyright © 2012 S. Karger AG, Basel

Introduction

Pulmonary fibrosis is a consequence of the pathophysiological response to injury that may be induced by a variety of factors, including medicinal agents, radiation, infection or exposure to toxic particles; however, the causes of idiopathic pulmonary fibrosis (IPF) remain unknown. IPF is a specific form of chronic interstitial pneumonia that is characterized by progressive fibrosis and destruction of the normal lung architecture. There is currently no established therapeutic strategy to prevent the development of pulmonary fibrosis [1]. Although the pathogenesis of pulmonary fibrosis is unknown, leukotrienes (LTs) have been implicated in the etiology of this disease. The LTs are a family of lipid mediators that are derived from arachidonic acid through the action of 5-lipoxygen-

ase (5-LOX). 5-LOX is an important enzyme in the production of leukotriene A₄ (LTA₄), an unstable precursor that is then converted to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase. LTC₄ is then further converted to LTD₄ and to LTE₄. LTB₄ exerts its biological effects via LTB₄ type 1 receptors and LTB₄ type 2 receptors. Cysteinyll leukotrienes (CysLTs), including LTC₄, LTD₄, and LTE₄, exert their effects by activating at least two subtypes of receptors: CysLT type 1 (CysLT₁) receptors and CysLT₂ receptors. LTB₄ is a chemoattractant for a variety of inflammatory cells, and it is also thought to be involved in various inflammatory diseases. CysLTs are known as proinflammatory mediators that promote bronchoconstriction, mucus secretion, tissue edema and leukocyte infiltration. CysLT₁ receptor antagonists have been used as therapeutic agents for asthma patients. In addition to proinflammatory activities, LTs have also been shown to contribute to the development of fibrosis by acting directly on fibroblasts or myofibroblasts to promote proliferation, collagen synthesis and chemotaxis [2–5]. Experiments using the bleomycin model of pulmonary fibrosis in mice have shown that depletion of LTs by disruption of the LTC₄ synthase or the 5-LOX genes attenuated the induction of pulmonary fibrosis [6, 7]. Furthermore, recent studies have shown that short-term treatment of mice with a CysLT₁ receptor antagonist for 2 weeks inhibited the development of bleomycin-induced pulmonary fibrosis [8, 9]. These results suggest that LTs participate in the pathogenesis of bleomycin-induced pulmonary fibrosis in mice.

Although the bleomycin model of pulmonary fibrosis in rodents is the most commonly used and well-characterized, there is no animal model recapitulating all of the features of human pulmonary fibrosis. Furthermore, several important points should be noted regarding the bleomycin model. Previous reports have shown that the fibrotic response to bleomycin reaches its peak at 3–4 weeks, whereas the resolution of fibrosis begins after this period and is completed as early as 6 weeks [10, 11]. Importantly, numerous agents have been shown to inhibit fibrosis in bleomycin-induced pulmonary fibrosis in rodents, yet most of these agents have been disappointing in the treatment of human IPF [1]. Although the bleomycin model is useful for investigating the fibrotic response to acute injury and for evaluating the effect of short-term treatment with various drugs in the early inflammatory stage, alternative models showing chronic and irreversible fibrosis are needed to further elucidate the pathogenesis of human pulmonary fibrosis. Silica-induced pulmonary fibrosis in mice is an experimental model of silicosis;

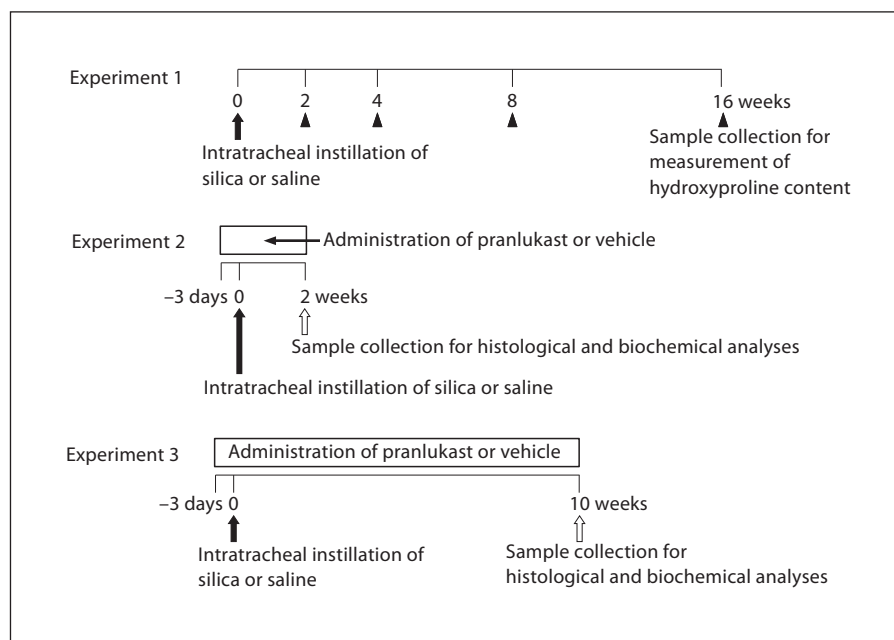
this fact notwithstanding, the persistence of the fibrotic stimulus in the silica-induced model seems advantageous for investigating the pathogenesis of pulmonary fibrosis [11]. Furthermore, we have previously shown histological similarities between the murine model of silica-induced pulmonary fibrosis and human pulmonary fibrosis, such as alveolar wall thickening, obliteration of alveolar space, scattered fibroblastic foci and a loss of architectural integrity [12]. Taken together, these observations suggest that the silica model may be more suitable than the bleomycin model for analyzing the pathogenesis of pulmonary fibrosis, especially in the chronic phase. Importantly, our previous study also showed that the lung tissue content of CysLTs and LTB₄ was increased in both the acute and the chronic phases of silica-induced pulmonary fibrosis [12]. These results suggest that LTs may contribute to the pathogenesis of silica-induced fibrosis, while pharmacological studies with specific antagonists of LT receptors have not been performed. To further elucidate the role for CysLTs in pulmonary fibrosis, we administered mice pranlukast, a selective CysLT₁ receptor antagonist, for 2 or 10 weeks after intratracheal silica instillation and investigated the effect of pranlukast on the progression of silica-induced pulmonary fibrosis.

Materials and Methods

Animal Preparation and Induction of Pulmonary Fibrosis

Female C57BL/6J mice (8 weeks of age; 16–20 g) were obtained from Charles River Japan (Kanagawa). Pulmonary fibrosis was induced by intratracheal instillation of crystalline silica particles as described previously [12]. The trachea of each animal was exposed under sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan) anesthesia, and 50 mg/kg of silica in 0.05 ml of sterile saline was instilled intratracheally. Three series of experiments were conducted as follows. First, we confirmed the persistent progression in silica-induced pulmonary fibrosis in mice (fig. 1: experiment 1). For this experiment, lobes of the lungs of silica-instilled mice were collected for the measurement of hydroxyproline content at 2, 4, 8 and 16 weeks after silica instillation (n = 6 for each time point). The lobes of the lungs of normal control mice were also collected at 2, 4, 8 and 16 weeks after the intratracheal instillation of sterile saline (n = 6 for each time point). We then investigated the effect of short-term treatment with pranlukast on the acute inflammatory response induced by silica instillation (fig. 1: experiment 2). Mice instilled with silica were divided into 2 experimental groups. One group (pranlukast-treated silica-induced pulmonary fibrosis mice: n = 10) received pranlukast orally (30 mg/kg/day, once a day; provided by Ono Pharmaceutical Co., Ltd., Osaka, Japan) via a gastric tube for 3 days before and 2 weeks after silica instillation. To gain steady drug effect at the moment of intratracheal silica instillation for inducing pulmonary fibrosis, we started oral pranlukast treatment 3 days

Fig. 1. Experimental protocols. Experiment 1: To confirm the persistent progression of silica-induced pulmonary fibrosis, lobes of the lungs of silica-instilled mice and saline-instilled control mice were collected at 2, 4, 8 and 16 weeks after silica or saline instillation, and the hydroxyproline content was measured. Experiment 2: To investigate the effect of short-term treatment with pranlukast on the acute inflammatory response, silica-instilled mice were treated with pranlukast or vehicle for 3 days before and for 2 weeks after silica instillation. Experiment 3: To investigate the effect of long-term treatment with pranlukast on the pathogenesis of silica-induced pulmonary fibrosis in the chronic phase, silica-instilled mice were treated with pranlukast or with vehicle for 3 days before and for 10 weeks after silica instillation. In experiments 2 and 3, saline-instilled control mice received the vehicle alone over the same period.



prior to the silica instillation. Pranlukast is a highly selective CysLT₁ receptor antagonist that has been developed in Japan for the treatment of asthma [13]. Since pranlukast is insoluble in water, it was suspended in 0.5% carboxymethylcellulose aqueous solution. Previous study showed that oral administration of 0.5% carboxymethylcellulose aqueous solution did not affect the baseline cytokine level in the mouse lung [14]. The other group (vehicle-treated silica-induced pulmonary fibrosis mice; $n = 10$) received the vehicle alone orally (0.5% carboxymethylcellulose aqueous solution) over the same period. Age-matched mice that received a single intratracheal instillation of sterile saline and daily oral treatment with vehicle served as normal controls ($n = 7$). We then investigated the effect of long-term treatment with pranlukast on the progression of silica-induced pulmonary fibrosis in mice (fig. 1: experiment 3). Mice instilled with silica were divided into 2 experimental groups. One group (pranlukast-treated silica-induced pulmonary fibrosis mice; $n = 12$) received pranlukast (30 mg/kg/day, once a day) for 3 days before and 10 weeks after silica instillation. The other group (vehicle-treated silica-induced pulmonary fibrosis mice; $n = 16$) received the vehicle alone over the same period. Age-matched mice that received a single intratracheal instillation of sterile saline and daily oral treatment with vehicle served as normal controls ($n = 8$). After treatment with pranlukast or vehicle, lobes of the lungs and bronchoalveolar lavage fluid (BALF) were collected for histological and biochemical analyses. To obtain all data from each mouse, we harvested the left lung for BALF collection and measurement of hydroxyproline content, the middle lobe of the right lung for measurement of mRNA levels, the inferior lobe of the right lung for histological analysis and the postcaval lobe of the right lung for measuring tissue LT content. This study was conducted in accordance with the guide for the Care and Use of Laboratory Animals prepared by the Shimane University School of Medicine, and the study pro-

ocols were reviewed and approved by the University's Committee on the Care and Use of Laboratory Animals.

Analysis of Inflammatory Cells in BALF

BALF was collected from the left lung by lavage with 1.5 ml (5 washes of 0.3 ml each) of PBS containing 5 mM EDTA. The numbers of total cells, macrophages, lymphocytes and neutrophils in BALF were counted as described previously [9, 12].

Measurement of LT Content in BALF and Lung Tissues

The LT content in BALF and lung tissues was measured as described previously [12]. Briefly, the supernatants from the centrifuged BALF and the homogenates of the postcaval lobe of the right lung were used for LT measurements. Lipids were extracted from the supernatants using methanol and C₁₈ Sep-Pak cartridges (Waters, Milford, Mass., USA). The CysLT and LT_{B4} content in BALF and in lung tissues was measured using enzyme-linked immunoassay (ELISA) kits (Cayman Chemicals, Ann Arbor, Mich., USA), according to the manufacturer's instructions. The ELISA kit for CysLTs detects LTC₄, LTD₄ and LTE₄.

Measurement of Hydroxyproline Content

The left lung was removed, lyophilized for 24 h, and treated with 1 ml of 6 N HCl (18 h, 120°C). The acid hydrolysates were then used for measuring hydroxyproline content as described previously [9, 12].

Histological Analysis

The inferior lobe of the right lung was carefully inflated with 10% buffered formalin at a constant pressure of 200 mm H₂O for 5 min and was then fixed with 10% buffered formalin. Lung tissues were embedded in paraffin, and 5-μm sections were subjected to hematoxylin and eosin (H&E) or Azan staining.

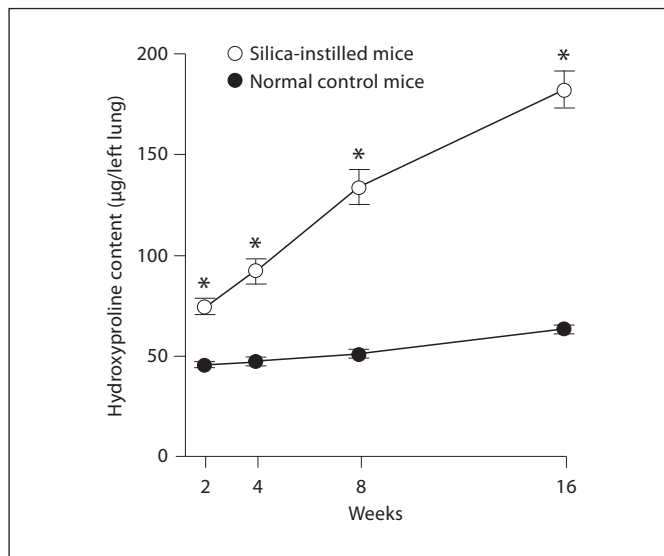


Fig. 2. Hydroxyproline content in the lung tissues of normal control mice (2, 4, 8 and 16 weeks after saline instillation) and of silica-instilled mice (2, 4, 8 and 16 weeks after silica instillation) was measured. Results are shown as the means $\pm$ SEM. * $p < 0.05$ versus normal mice.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from the middle lobe of the right lung. The mRNA levels for TGF- β 1 and TNF- α were measured by quantitative RT-PCR as described previously [9, 12].

Statistical Analysis

All data are expressed as the means $\pm$ SEM. For the unpaired test, comparisons between 2 groups were made using Student's *t* test. For multiple comparisons, data were examined by analysis of variance (ANOVA) followed by Bonferroni's/Dunn's post hoc test. Differences were considered statistically significant at a level of $p < 0.05$.

Results

Sustained Increase of Hydroxyproline Content in Lung Tissues after Silica Instillation

The hydroxyproline content in lung tissues was significantly increased by 2 weeks after silica instillation and continued to increase until the end of the experiment at 16 weeks ($p < 0.05$; fig. 2). This result indicates that the silica-induced pulmonary fibrosis persisted for at least the duration of experimental period. Therefore, silica-induced pulmonary fibrosis in mice is a useful model for analyzing the pathogenesis of pulmonary fibrosis in the chronic phase.

Table 1. Effects of pranlukast treatment on the number of inflammatory cells in BALF (10^4 cells/left lung)

	Total cells	Macrophages	Lymphocytes	Neutrophils
<i>2 weeks</i>				
Normal	4.2 ± 0.7	4.0 ± 0.7	0.2 ± 0.06	0.001 ± 0.001
Vehicle	$31.4 \pm 5.2^*$	$17.5 \pm 3.1^*$	$7.5 \pm 1.3^*$	$6.4 \pm 1.2^*$
Pranlukast	$33.6 \pm 10.2^*$	$20.0 \pm 6.7^*$	$8.1 \pm 2.3^*$	$5.5 \pm 1.4^*$
<i>10 weeks</i>				
Normal	3.7 ± 0.4	3.9 ± 0.5	0.1 ± 0.02	0.0 ± 0.0
Vehicle	$19.4 \pm 3.0^{*,**}$	$8.7 \pm 1.5^{*,**}$	$4.7 \pm 0.6^{*,**}$	$5.5 \pm 1.1^*$
Pranlukast	$18.8 \pm 1.6^*$	$9.5 \pm 1.0^*$	$4.9 \pm 0.6^*$	$4.3 \pm 0.5^*$

The numbers of total cells, macrophages, lymphocytes and neutrophils were counted in BALF from vehicle-treated (vehicle) and pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mice, and normal control mice (normal) 2 and 10 weeks after silica instillation. The results are shown as the means $\pm$ SEM.

* $p < 0.05$ versus age-matched normal group.

** $p < 0.05$ versus vehicle group at 2 weeks. There was no difference between the vehicle group and the pranlukast group at either 2 or 10 weeks after silica instillation.

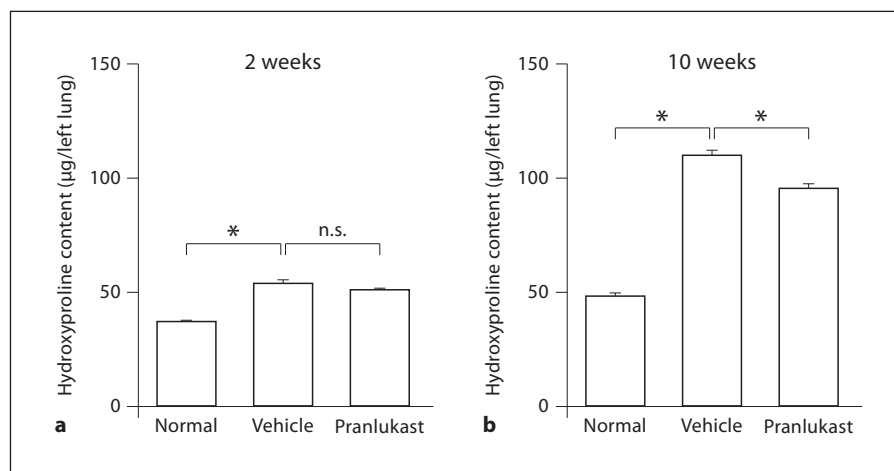
Effects of Pranlukast on the Number of Inflammatory Cells in BALF

Two weeks after silica instillation, the numbers of total cells, macrophages, lymphocytes and neutrophils in the BALF were markedly increased in vehicle-treated pulmonary fibrosis mice compared with those in age-matched normal control mice ($p < 0.05$; table 1). At 10 weeks, the numbers of inflammatory cells in the BALF of vehicle-treated pulmonary fibrosis mice were still higher than those of age-matched normal control mice ($p < 0.05$; table 1); however, the numbers of inflammatory cells (except neutrophils) at 10 weeks were lower than those at 2 weeks ($p < 0.05$; table 1). Pranlukast did not affect the numbers of inflammatory cells either 2 or 10 weeks after silica instillation (table 1).

Effect of Pranlukast on the Hydroxyproline Content in Lung Tissues

Two weeks after silica instillation, the hydroxyproline content in lung tissues was significantly increased in vehicle-treated pulmonary fibrosis mice compared with that in normal control mice, but was not affected by pranlukast treatment ($p < 0.05$; fig. 3a). Ten weeks after silica instillation, the hydroxyproline content in lung tissues was markedly increased in vehicle-treated pulmonary fibrosis mice compared with that in normal control mice, and pranlukast treatment effectively attenuated the observed increase in the hydroxyproline content in the fibrotic lungs ($p < 0.05$; fig. 3b).

Fig. 3. Effect of pranlukast on hydroxyproline content in lung tissues. Two (a) and 10 (b) weeks after silica instillation, the hydroxyproline content was measured in the lung tissues of vehicle-treated (vehicle) and pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mice, and normal control mice (normal). Results are shown as the means $\pm$ SEM. * $p < 0.05$.



Effects of Pranlukast on Histopathological Changes

Two weeks after silica instillation, alveolar wall thickening, infiltration of inflammatory cells into the alveolar spaces and scattered nodules were observed in fibrotic lung tissues (fig. 4a, b). Nodular regions were surrounded by relatively unaffected lung tissue. The fibrotic response, which was identified by a blue color in Azan staining, appeared in the nodular regions 2 weeks after silica instillation (fig. 4b). Treatment with pranlukast for 2 weeks had no effect on these histological changes (fig. 4e, f). Ten weeks after silica instillation, the nodules become less-inflammatory dense fibrotic lesions characterized by a massive deposition of extracellular matrix, loss of architectural integrity and a severe hyperplasia of fibroblast-like cells, alveolar macrophage-like cells and type II alveolar epithelial-like cells (fig. 4c, d). The degree of pulmonary fibrosis was extensive 10 weeks after silica instillation (fig. 4d), and pranlukast treatment during this period attenuated the formation of these dense fibrotic foci and the progression of fibrosis in the silica-instilled mice (fig. 4g, h).

Effects of Pranlukast on the CysLT and LTB₄ Content in BALF and Lung Tissues

Two weeks after silica instillation, the content of CysLTs and LTB₄ in BALF and lung tissues was significantly increased in vehicle-treated pulmonary fibrosis mice compared with that in normal control mice ($p < 0.05$; fig. 5a, c, 6a, c), but was not affected by pranlukast treatment (fig. 5a, c, 6a, c). Ten weeks after silica instillation, the content of CysLTs and LTB₄ in BALF and lung tissues was further increased in vehicle-treated pulmonary fibrosis mice compared with that in normal control

mice ($p < 0.05$; fig. 5b, d, 6b, d). Pranlukast treatment for 10 weeks decreased the CysLT content in both BALF and lung tissues ($p < 0.05$; fig. 5b, d) and decreased the LTB₄ content in the BALF ($p < 0.05$; fig. 6b). Although it decreased the LTB₄ content in lung tissues, the difference was not significant between vehicle-treated and pranlukast-treated pulmonary fibrosis mice (fig. 6d). We used the left lung for measuring the LT content in BALF and used the postcaval lobe of the right lung for measuring the LT content in tissues. The two experimental procedures are different; therefore, it is not reasonable to simply compare the LT content in BALF with that in tissues.

Effects of Pranlukast on the mRNA Levels of TGF- β 1 and TNF- α in Lung Tissues

Two and 10 weeks after silica instillation, the mRNA levels of TGF- β 1 and TNF- α were significantly increased in vehicle-treated pulmonary fibrosis mice compared to those in normal control mice ($p < 0.05$; fig. 7a–d), but were not affected by pranlukast treatment for either 2 weeks or 10 weeks (fig. 7a–d).

Discussion

One of the most important findings of our study is that long-term treatment with pranlukast for 10 weeks significantly attenuated the development of pulmonary fibrosis, but short-term treatment, for 2 weeks, failed to inhibit the initial increase in hydroxyproline content in the fibrotic lungs. These results show that the beneficial antifibrotic effect of pranlukast, which was observed after the 10-week treatment, may be due to the inhibition

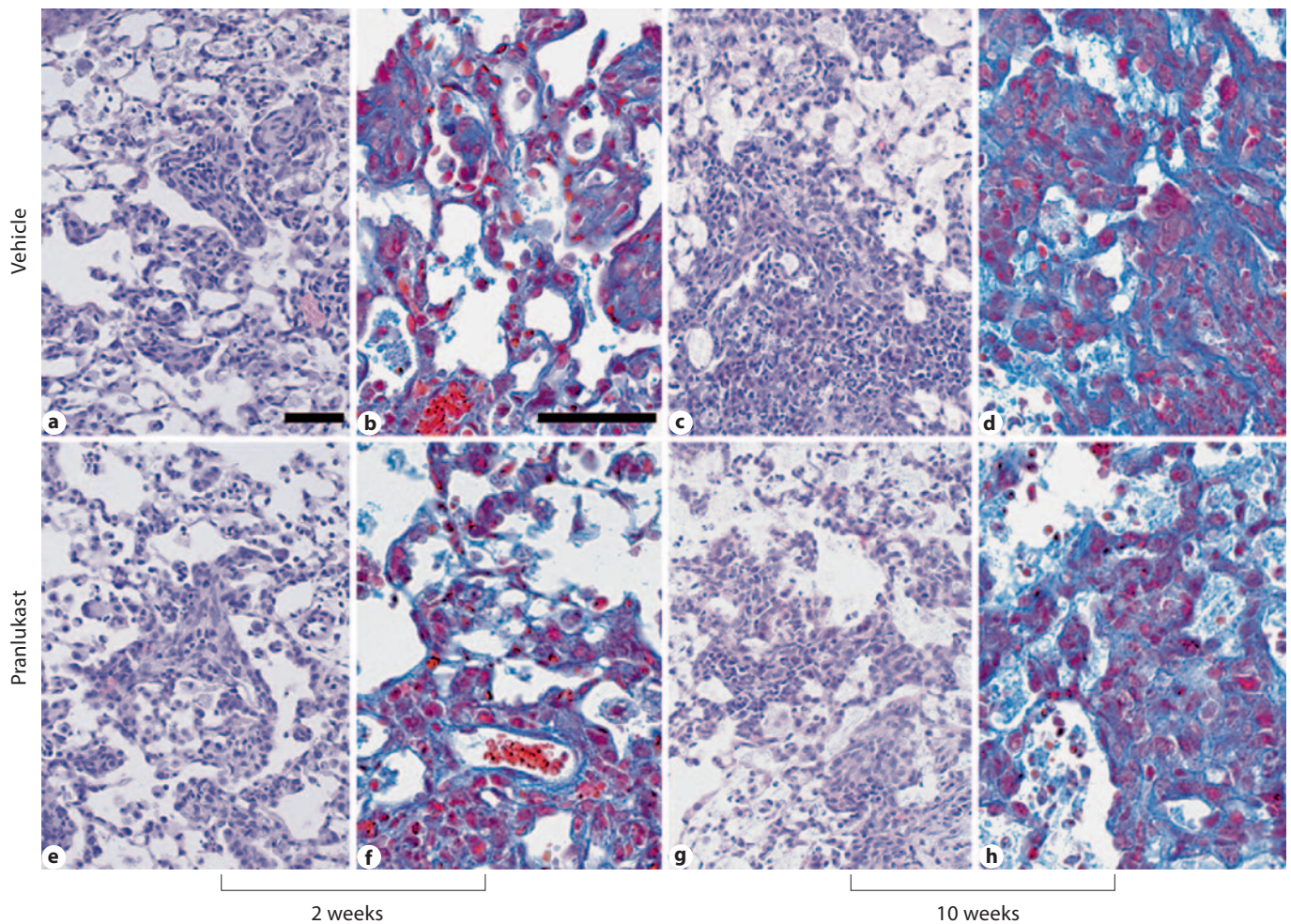


Fig. 4. Effects of pranlukast on histopathological changes. Lung tissue cross-sections stained with H&E (**a, c, e, g**) and Azan (**b, d, f, h**) from a vehicle-treated (vehicle) and a pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mouse. H&E- and Azan-stained sections of a vehicle-treated (**a, b**) and a pranlukast-

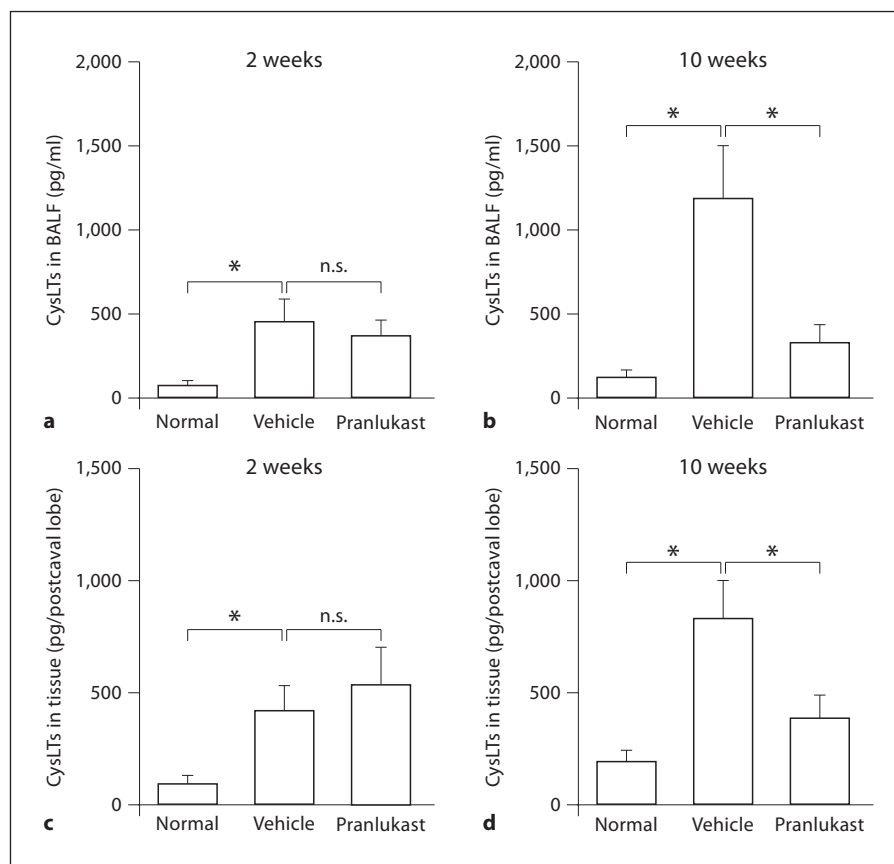
treated (**e, f**) pulmonary fibrosis mouse 2 weeks after silica instillation. H&E- and Azan-stained sections of a vehicle-treated (**c, d**) and a pranlukast-treated (**g, h**) pulmonary fibrosis mouse 10 weeks after silica instillation. In Azan-stained sections, the blue color indicates the presence of collagen. Scale bar indicates 50 μ m.

of the progression of fibrosis rather than the inhibition of its onset. In addition, the number of inflammatory cells in BALF, the LT content and the mRNA levels of cytokines were not affected by the 2-week treatment; thus, pranlukast treatment has no effect on the acute inflammatory response. Interestingly, recent evidence has demonstrated that inflammation might not play a pivotal role in the pathogenesis of human IPF or in silica-induced pulmonary fibrosis in mice [1, 15, 16]. Although further detailed pharmacological studies are necessary to determine whether the acute inflammatory response affects the progression of pulmonary fibrosis in the chronic phase of the disease, our results show that pranlukast

treatment has antifibrotic effects that are independent of the effect on this response.

Another important new finding is that the content of CysLTs in fibrotic lungs was markedly increased in silica-instilled mice, and that the 10-week pranlukast treatment attenuated both the progression of pulmonary fibrosis and the observed increases in CysLT content in fibrotic lungs. Interestingly, a clinical study also showed LTC₄ levels to be significantly increased in lung homogenates taken from patients with IPF [17]. Although CysLTs are known as proinflammatory mediators, several in vitro studies have shown that they act directly on fibroblasts or myofibroblasts to promote their migration, proliferation

Fig. 5. Effects of pranlukast on CysLT content in BALF and lung tissues. Two (**a, c**) and 10 (**b, d**) weeks after silica instillation, the CysLT content in the BALF (**a, b**) and lung tissues (**c, d**) of vehicle-treated (vehicle) and pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mice, and normal control mice (normal) was measured by ELISA. Results are shown as the means $\pm$ SEM. * $p < 0.05$.



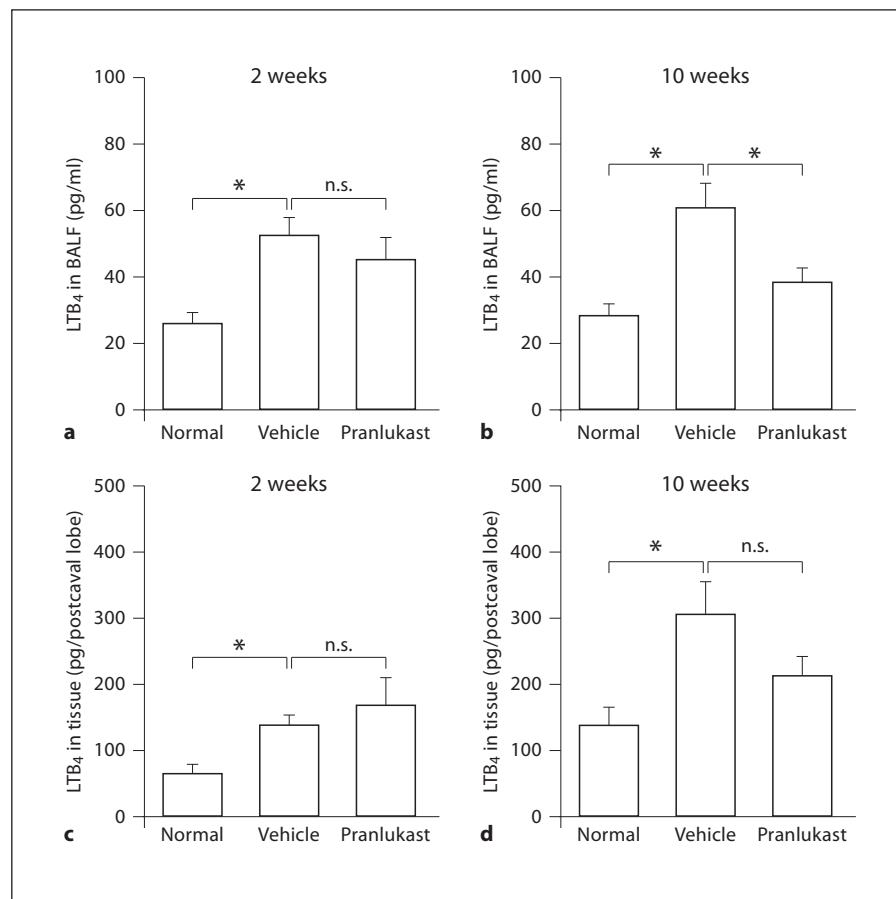
and collagen synthesis. LTC_4 induced both the mitogenesis of human lung fibroblasts [3] and the proliferation of myofibroblasts derived from patients with interstitial lung disease [2], and LTC_4 and LTD_4 stimulated collagen synthesis in rat lung fibroblasts [4]. Furthermore, LTD_4 promoted the proliferation of murine and human bone marrow-derived fibrocytes, which participate in tissue remodeling and repair [18]. These profibrotic effects of CysLTs were blocked by treatment with a CysLT₁ receptor antagonist [2, 3, 18]. Taken together, the increased production of CysLTs in the fibrotic lungs of silica-instilled mice may be involved in the pathogenesis of pulmonary fibrosis, and pranlukast treatment may exert its antifibrotic effect by antagonizing the profibrotic effect of CysLTs and decreasing their production in fibrotic lungs.

Although the precise mechanism by which pranlukast reduces CysLTs in the lung is unclear, inhibition of CysLT₁ receptor-mediated responses by CysLT₁ receptor antagonists may modulate CysLT production. Previous in vitro studies have shown that LTC_4 activates the calcium release-activated calcium (CRAC) channel, and

that ERK activation following Ca^{2+} entry through CRAC channels is functionally important because it is required for the activation of both cPLA₂ and 5-LOX, both of which are essential for the synthesis of LTs [19]. This positive feedback mechanism for the production of LTC_4 was inhibited by pretreatment with montelukast, a CysLT₁ receptor antagonist [19]. In addition, recent evidence has shown that CysLT₁ receptor antagonists including pranlukast are able to reduce the extracellular content of CysLTs in a CysLT₁ receptor-independent manner. Pranlukast and other CysLT₁ receptor antagonists, such as MK-571 and montelukast, reportedly inhibit multidrug resistance proteins that are known to transport LTC_4 [20–22].

We also show that TGF- β 1 and TNF- α are increased in our model of silica-induced pulmonary fibrosis. Previous studies have shown that treatment with TGF- β -neutralizing antibodies prevented bleomycin-induced pulmonary fibrosis in mice [23], and that gene transfer of active TGF- β 1 induced severe pulmonary fibrosis in rats [24]. Furthermore, treatment with recombinant sol-

Fig. 6. Effects of pranlukast on LTB₄ content in BALF and lung tissues. Two (**a, c**) and 10 (**b, d**) weeks after silica instillation, the LTB₄ content in the BALF (**a, b**) and the lung tissues (**c, d**) of vehicle-treated (vehicle) and pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mice, and normal control mice (normal) was measured by ELISA. Results are shown as the means $\pm$ SEM. * $p < 0.05$.

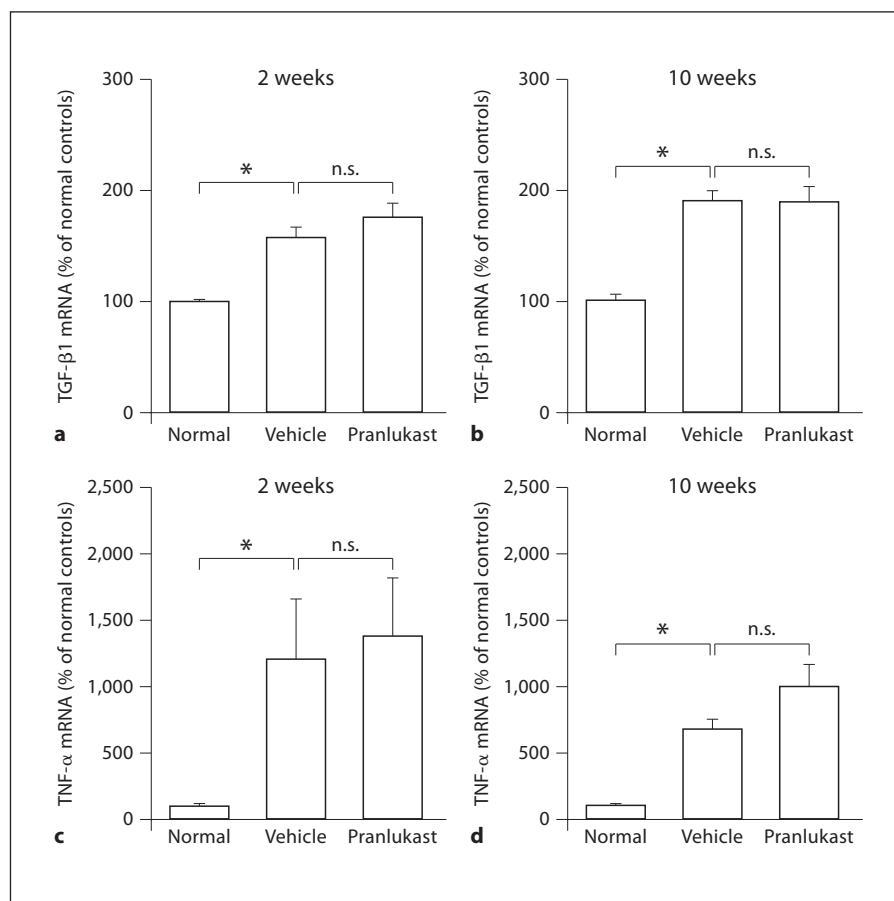


uble TNF- α receptor suppressed fibrosis in both silica-induced and bleomycin-induced pulmonary fibrosis in mice [25]. These results suggest that the increased expression of TGF- β 1 and TNF- α can induce pulmonary fibrosis. In addition, previous studies with a bleomycin model showed that the treatment with a CysLT₁ receptor antagonist for 2 weeks decreased the protein content of TGF- β 1 in BALF or TGF- β 1 expression in lung tissues and inhibited the development of pulmonary fibrosis [8, 9]. Furthermore, several in vitro studies have shown that CysLTs induce the production of TGF- β 1 in cultured human airway epithelial cells and type II pneumocytes [3, 26], and also induce TNF- α production in cultured alveolar macrophages [27]. Therefore, CysLTs may induce fibrosis through activating profibrotic cytokines, such as TGF- β 1 and TNF- α . Nevertheless, in this study, pranlukast treatment failed to attenuate the expression of TGF- β 1 and TNF- α after either 2 or 10 weeks of treatment. Although the mechanism is unclear, CysLTs may not play a dominant regulatory role for these cytokines in

silica-induced pulmonary fibrosis. To date, their profibrotic effects have been thought to be related to the increased production of major profibrotic cytokines, such as TGF- β 1 and TNF- α . However, our results show that CysLTs directly exert profibrotic effects in the lungs of silica-instilled mice without activating the expression of major profibrotic cytokines.

Although pranlukast treatment for 2 weeks affected neither CysLT nor LTB₄ production in the fibrotic lung, 10-week treatment decreased not only CysLT but also LTB₄ production. The LTB₄ content in BALF and in fibrotic lung tissues was markedly increased in the chronic phase of disease after silica instillation, while pranlukast treatment significantly attenuated the LTB₄ content in the BALF, and there was a trend toward decrease in LTB₄ content in the lung tissues as well. LTB₄ is a well-known chemoattractant for neutrophils and for other inflammatory cells, yet there are few studies investigating its profibrotic effect. A previous in vitro study showed that it is also a chemoattractant for fibroblasts [5], and a

Fig. 7. Effects of pranlukast on mRNA levels of TGF- β 1 and TNF- α . Two (**a, c**) and 10 (**b, d**) weeks after silica instillation, the mRNA levels of TGF- β 1 (**a, b**) and TNF- α (**c, d**) in the lung tissues of vehicle-treated (vehicle) and pranlukast-treated (pranlukast) silica-induced pulmonary fibrosis mice, and normal control mice (normal) were measured by quantitative RT-PCR analysis. Results are shown as the means $\pm$ SEM. * $p < 0.05$.



recent study showed that treatment with an LTB₄ receptor antagonist for 3 weeks inhibited the development of bleomycin-induced pulmonary fibrosis in mice [28]. These studies suggest that LTB₄ is involved in the pathogenesis of pulmonary fibrosis. In our study, therefore, a reduction of LTB₄ content by pranlukast treatment may also have affected the progression of silica-induced pulmonary fibrosis.

Inflammatory cells, such as macrophages and neutrophils, that migrate into the alveolar space after silica instillation, are well known to produce CysLTs and LTB₄ [29–31]. In addition to these inflammatory cells, lung fibroblasts and type II alveolar epithelial cells also produce both CysLTs and LTB₄ [32, 33]. The characteristic pathological finding in silica-induced pulmonary fibrosis is the formation of silicotic nodules, which consist of fibroblast-like cells, type II alveolar epithelial-like cells, and alveolar macrophage-like cells [12]. In the chronic phase, the number of inflammatory cells in BALF was lower than that in the acute phase, and the nodular regions became

less-inflammatory and dense fibrotic foci. Importantly, long-term treatment with pranlukast attenuated the formation of dense fibrotic foci and the production of LTs in fibrotic lungs, but not the number of inflammatory cells in the BALF in the chronic phase of the disease. Therefore, in the chronic phase, cells that proliferate in fibrotic foci may contribute more to LT production than the recruited inflammatory cells. LTs produced locally in fibrotic foci may play an important role in the progression of silica-induced pulmonary fibrosis.

In this study, we measured the sum of CysLTs including LTC₄, LTD₄ and LTE₄. Because LTC₄ is rapidly converted to LTD₄ and LTE₄, it is technically difficult to separately analyze the role of each CysLT in vivo. In addition, LTD₄ has the highest affinity to CysLT₁ receptor, but LTC₄ and LTE₄ also bind weakly to it. Therefore, to investigate the effect of pranlukast in vivo, it is rational to evaluate the CysLTs as a whole. However, CysLTs exert their effects not only via the CysLT₁ receptor but also via the CysLT₂ receptor, and a recent study suggests

that LTE₄ induces skin edema by activating a novel receptor for LTE₄ [34]. Hereafter, further detailed studies with methods that allow measurement of each CysLT separately, as well as studies with selective agonists or antagonists for the CysLT₂ receptor and for novel receptors, will be indispensable to explore the precise role of each CysLT in the pathogenesis of pulmonary fibrosis.

In conclusion, we have shown that long-term treatment with pranlukast, a selective CysLT₁ receptor antagonist, attenuates the progression of silica-induced pulmonary fibrosis in mice in the chronic phase, but that short-term treatment has no effect on the onset of pulmonary fibrosis in the acute phase. Furthermore, the content of LTs in fibrotic lungs in the chronic phase of disease

progression was markedly increased in silica-instilled mice and long-term pranlukast treatment attenuated the progression of pulmonary fibrosis while concurrently decreasing the LT content in fibrotic lungs without affecting the expression of profibrotic cytokines. Therefore, the increased production of LTs in the fibrotic lungs may contribute directly to the progression of pulmonary fibrosis, especially in the chronic phase.

Acknowledgments

This work was supported in part by Grant-in-Aid for Young Scientists (B) No. 22790756 (C.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- 1 du Bois RM: Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug Discov* 2010;9:129–140.
- 2 Fireman E, Schwartz Y, Mann A, Greif J: Effect of montelukast, a cysteinyl receptor antagonist, on myofibroblasts in interstitial lung disease. *J Clin Immunol* 2004;24:418–425.
- 3 Perng DW, Wu YC, Chang KT, Wu MT, Chiou YC, Su KC, Perng RP, Lee YC: Leukotriene C₄ induces TGF-β1 production in airway epithelium via p38 kinase pathway. *Am J Respir Cell Mol Biol* 2006;34:101–107.
- 4 Phan SH, McGarry BM, Loeffler KM, Kunkel SL: Binding of leukotriene C₄ to rat lung fibroblasts and stimulation of collagen synthesis in vitro. *Biochemistry* 1988;27:2846–2853.
- 5 Mensing H, Czarnetzki BM: Leukotriene B₄ induces in vitro fibroblast chemotaxis. *J Invest Dermatol* 1984;82:9–12.
- 6 Beller TC, Friend DS, Maekawa A, Lam BK, Austen KF, Kanaoka Y: Cysteinyl leukotriene 1 receptor controls the severity of chronic pulmonary inflammation and fibrosis. *Proc Natl Acad Sci USA* 2004;101:3047–3052.
- 7 Peters-Golden M, Bailie M, Marshall T, Wilke C, Phan SH, Toews GB, Moore BB: Protection from pulmonary fibrosis in leukotriene-deficient mice. *Am J Respir Crit Care Med* 2002;165:229–235.
- 8 Izumo T, Kondo M, Nagai A: Cysteinyl-leukotriene 1 receptor antagonist attenuates bleomycin-induced pulmonary fibrosis in mice. *Life Sci* 2007;80:1882–1886.
- 9 Shimbori C, Shiota N, Okunishi H: Effects of montelukast, a cysteinyl-leukotriene type 1 receptor antagonist, on the pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Eur J Pharmacol* 2011;650:424–430.
- 10 Lawson WE, Polosukhin VV, Stathopoulos GT, Zoia O, Han W, Lane KB, Li B, Donnelly EF, Holburn GE, Lewis KG, Collins RD, Hull WM, Glasser SW, Whitsett JA, Blackwell TS: Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. *Am J Pathol* 2005;167:1267–1277.
- 11 Moore BB, Hogaboam CM: Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L152–L160.
- 12 Shimbori C, Shiota N, Okunishi H: Involvement of leukotrienes in the pathogenesis of silica-induced pulmonary fibrosis in mice. *Exp Lung Res* 2010;36:292–301.
- 13 Obata T, Okada Y, Motoishi M, Nakagawa N, Terawaki T, Aishita H: In vitro antagonism of ONO-1078, a newly developed anti-asthma agent, against peptide leukotrienes in isolated guinea pig tissues. *Jpn J Pharmacol* 1992;60:227–237.
- 14 Okunishi K, Dohi M, Nakagome K, Tanaka R, Yamamoto K: A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. *J Immunol* 2004;173:6393–6402.
- 15 Rabolli V, Re SL, Uwambayinema F, Yakoub Y, Lison D, Huaux F: Lung fibrosis induced by crystalline silica particles is uncoupled from lung inflammation in NMRI mice. *Toxicol Lett* 2011;203:127–134.
- 16 Selman M, King TE, Pardo A, American Thoracic Society, European Respiratory Society, American College of Chest Physicians: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001;134:136–151.
- 17 Wilborn J, Bailie M, Coffey M, Burdick M, Strieter R, Peters-Golden M: Constitutive activation of 5-lipoxygenase in the lungs of patients with idiopathic pulmonary fibrosis. *J Clin Invest* 1996;97:1827–1836.
- 18 Vannella KM, McMillan TR, Charbeneau RP, Wilke CA, Thomas PE, Toews GB, Peters-Golden M, Moore BB: Cysteinyl leukotrienes are autocrine and paracrine regulators of fibrocyte function. *J Immunol* 2007;179:7883–7890.
- 19 Di Capite J, Shirley A, Nelson C, Bates G, Parekh AB: Inter cellular Ca²⁺ wave propagation involving positive feedback between CRAC channels and cysteinyl leukotrienes. *FASEB J* 2009;23:894–905.
- 20 Nakano R, Oka M, Nakamura T, Fukuda M, Kawabata S, Terashi K, Tsukamoto K, Noguchi Y, Soda H, Kohno S: A leukotriene receptor antagonist, ONO-1078, modulates drug sensitivity and leukotriene C₄ efflux in lung cancer cells expressing multidrug resistance protein. *Biochem Biophys Res Commun* 1998;251:307–312.
- 21 Roy U, Chakravarty G, Honer Zu Bentrup K, Mondal D: Montelukast is a potent and durable inhibitor of multidrug resistance protein 2-mediated efflux of taxol and saquinavir. *Biol Pharm Bull* 2009;32:2002–2009.
- 22 Rius M, Hummel-Eisenbeiss J, Keppler D: ATP-dependent transport of leukotrienes B₄ and C₄ by the multidrug resistance protein ABCC4 (MRP4). *J Pharmacol Exp Ther* 2008;324:86–94.
- 23 Giri SN, Hyde DM, Hollinger MA: Effect of antibody to transforming growth factor β on bleomycin induced accumulation of lung collagen in mice. *Thorax* 1993;48:959–966.

- 24 Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor- β 1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100:768–776.
- 25 Piguet PF, Vesin C: Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. *Eur Respir J* 1994;7:515–518.
- 26 Bossé Y, Thompson C, McMahon S, Dubois CM, Stankova J, Rola-Pleszczynski M: Leukotriene D₄-induced, epithelial cell-derived transforming growth factor β 1 in human bronchial smooth muscle cell proliferation. *Clin Exp Allergy* 2008;38:113–121.
- 27 Ménard G, Bissonnette EY: Priming of alveolar macrophages by leukotriene D₄: potentiation of inflammation. *Am J Respir Cell Mol Biol* 2000;23:572–577.
- 28 Izumo T, Kondo M, Nagai A: Effects of a leukotriene B₄ receptor antagonist on bleomycin-induced pulmonary fibrosis. *Eur Respir J* 2009;34:1444–1451.
- 29 Koren HS, Joyce M, Devlin RB, Becker S, Driscoll K, Madden MC: Modulation of eicosanoid production by human alveolar macrophages exposed to silica in vitro. *Environ Health Perspect* 1992;97:77–83.
- 30 Sala A, Zarini S, Folco G, Murphy RC, Henson PM: Differential metabolism of exogenous and endogenous arachidonic acid in human neutrophils. *J Biol Chem* 1999;274:28264–28269.
- 31 Charbeneau RP, Peters-Golden M: Eicosanoids: mediators and therapeutic targets in fibrotic lung disease. *Clin Sci* 2005;108:479–491.
- 32 Cott GR, Westcott JY, Voelkel NF: Prostaglandin and leukotriene production by alveolar type II cells in vitro. *Am J Physiol* 1990;258:L179–L187.
- 33 James AJ, Penrose JF, Cazaly AM, Holgate ST, Sampson AP: Human bronchial fibroblasts express the 5-lipoxygenase pathway. *Respir Res* 2006;7:102.
- 34 Austen KF, Maekawa A, Kanaoka Y, Boyce JA: The leukotriene E₄ puzzle: finding the missing pieces and revealing the pathobiologic implications. *J Allergy Clin Immunol* 2009;124:406–414.