Cysteinyl-leukotriene 1 receptor antagonist attenuates bleomycin-induced pulmonary fibrosis in mice

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Abstract

Leukotrienes are lipid mediators of inflammation derived from the 5-lipoxygenase pathway of arachidonic acid metabolism, and recent evidence suggests that they play an important role in pulmonary fibrosis. Montelukast is a cysteinyl-leukotriene 1 receptor antagonist that has been found to reduce airway remodeling, including subepithelial fibrosis, in a murine model of asthma, but the therapeutic effect of montelukast on pulmonary fibrosis remains unclear. In this study, we investigated whether montelukast is capable of preventing bleomycin-induced pulmonary fibrosis in mice. On day 1, C57BL/6 mice were given a single intratracheal injection of bleomycin (2.5 mg/kg), and montelukast (1.0 mg/kg) or vehicle alone subcutaneously two hours later and on days 1-5 of each week for two weeks. The total number of cells in bronchoalveolar lavage fluid (BALF) was reduced in the montelukast group on day 7 and on day 14, and cellular inflammation and fibrosis were attenuated on day 14 as indicated by significant decrease in the Ashcroft score and lung hydroxyproline content. Although cysteinyl-leukotriene level in BALF was not significantly different, transforming growth factor β (TGFβ) level in BALF by ELISA and TGFβ expression in lung tissue by immunohistochemistry was reduced on day 14 in the montelukast group. The results of this study show that montelukast inhibits the inflammatory process and development of bleomycin-induced pulmonary fibrosis in mice and that these effects may be associated with a decrease in TGFβ expression. They also suggest that montelukast may serve as a new therapy for patients with interstitial pulmonary fibrosis. (247 words)

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and life-threatening disease, and the median survival time of patients is about four to five years after the onset of symptoms. IPF is characterized by accumulation of inflammatory cells, fibroblast proliferation and deposition of extracellular matrix (ECM) in the lungs. Corticosteroids and other immunosuppressive agents have been used to treat IPF, but their efficacy has been disappointing, and new insights into the pathobiology and establishment of a new therapy are urgently needed.

Leukotrienes (LTs) are lipid mediators of inflammation derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. Patients with IPF produce excessive amounts of LTB4 and cysteinyl LTs (cysLTs) in their lungs (Wilborn et al., 1996), and lower amounts of collagen and hydroxyproline are observed in the 5-LO gene knockout mice after administration of bleomycin (Peters-Golden et al., 2002). Because LTs have direct effect on migration, proliferation, and matrix protein synthesis by fibroblasts, LTs may play an important role in pulmonary fibrosis (Baud et al., 1987; Phan et al., 1988) and may be considered target molecules for the treatment of pulmonary fibrosis.

Montelukast is one of the cysteinyl-leukotriene1 (CysLT1) receptor antagonists that have been widely used in the treatment of asthma. It has been found to reduce airway remodeling, including subepithelial fibrosis, in a murine model of asthma (Henderson et al., 2002), and recent evidence suggests that montelukast inhibits LTC4-induced transforming growth factor β (TGFβ) production in airway epithelial cells (Perng et al., 2006). TGFβ is one of most important growth factors that cause excessive production of ECM protein and fibroblast proliferation, and since treatment of animals with TGFβ neutralizing antibodies prevents bleomycin-induced lung fibrosis (Giri et al., 1997), we sought to investigate

whether montelukast is capable of preventing bleomycin-induced pulmonary fibrosis in mice, and the role of TGFβ in the mechanism.

Materials and Methods

Animals

The animal protocol was approved by the Animal Care and Use Committee of Tokyo Women's Medical University. We used six-week-old C57BL/6 male mice. On day 1, the mice were given a single intratracheal injection of 50 μl of saline containing bleomycin (2.5 mg/kg) (Nippon Kayaku, Tokyo, Japan) or 50 μl of saline alone. Montelukast sodium (1.0 mg/kg) (Merck and Co., Inc., Rahway, NJ) dissolved in distilled water or vehicle alone was injected subcutaneously two hours later after the injection and on days 1-5 each week for two weeks. Thus, there were three groups of mice in this study: saline-injected mice given vehicle (non-treated group, n = 6-7), bleomycin-injected mice given vehicle (bleomycin group, n = 6-10), and bleomycin-injected mice given montelukast (montelukast group, n = 6-10).

We evaluated the severity of lung inflammation by bronchoalveolar lavage fluid (BALF) analysis on day 7 and on day 14, and we evaluated the effect of montelukast on pulmonary fibrosis by histological evaluation according to the Ashcroft score and the hydroxyproline content of the lungs on day 14.

BALF analysis

Mice were anesthetized with pentobarbital (50 mg/kg, ip), a tracheotomy was performed and a custom-built cannula was inserted. The lungs were lavaged with 1.0 ml of saline or PBS and then with 0.8 ml. The BALF was centrifuged at 360 *g* for 10 min, and the supernatant for the measurement of TGFβ was stored at -80˚C. A total cell count was performed

manually with a hemocytometer. Slides of BALF cells were prepared with cytospin, stained with May-Grünwald-Giemza stain, and ~1000 cells per sample were differentiated. CysLTs level in BALF on day 14 was measured by the enzymeimmunoassay system (Amersham Biosciences, Piscataway, NJ) as reported previously (Nagase et al., 2002). The detection limit of the assay was 10 pg/ml. TGFβ1 level in BALF on day 14 was measured with a mouse TGFβ1 ELISA kit (R&D system, Minneapolis, MN). The detection limit was 4.2 pg/ml.

Histological analysis

The left lung was fixed by inflation with 4% paraformaldehyde and embedded in paraffin (non-treated group, n = 6, bleomycin and montelukast group, n = 10). Sections were cut at 5 μm thick, and stained with hematoxylin-eosin. The Ashcroft score was used for semiquantitative analysis of fibrotic change as reported previously (Suzuki et al., 2003).

Immunostaining for TGFβ

The sections were deparaffinized in xylene and dehydrated in ethanol. They were washed three times with phosphate-buffered saline (PBS), reacted with peroxidase-blocking solution (DakoCytomation, A/S, Denmark) for 10 minutes at room temperature to block endogenous peroxidase activity, and then washed three times with PBS. Next, they were reacted with Protein Block Serum-Free (DakoCytomation) for 10 minutes at room temperature. Some sections were incubated at 4˚C overnight with anti-mouse TGFβ antibody (diluted 1:50; Santa Cruz Biotechnology. Inc, San Diego, CA), and control sections were incubated at 4˚C over night with a rabbit immunoglobulin fraction as a negative control (diluted 1:1000; DakoCytomation). The next day, all sections were washed three times with PBS. Antibody

that had bound to TGFβ was detected by incubation for 30 minutes at room temperature with dextran polymer reagent conjugated with peroxidase and secondary antibody (DAKO EnVision+, DakoCytomation). The sections were then washed three times with PBS, and color development was achieved by exposure to 3,3'-diaminobenzidine (DAKO DAB+ Liquid System, DakoCytomation) for 2 minutes. The tissues were counterstained with Mayer's hematoxylin.

Hydroxyproline assays

Lung homogenates were prepared and assayed for hydroxyproline content as previously described (Moore et al., 2000; Thrall et al., 1979).

Statistical analysis.

Data are reported as means ± SE. Statistical analysis was performed by one-way ANOVA followed by Sheffe's F test as a post hoc analysis test. P values of less than 0.05 were considered significant.

Results

Cell analysis of BALF

The total cell counts in BALF were markedly higher in the bleomycin group than in the non-treated group, and the counts on day 7 and day 14 showed that the increase was significantly inhibited by montelukast (bleomycin group vs. motelukast group, day 7: $1.8 \times 10^6 \pm 2.7 \times 10^5$ vs. $7.4 \times 10^5 \pm 5.9 \times 10^4$, $p < 0.05$; day 14: $1.8 \times 10^6 \pm 9.9 \times 10^4$ vs. $1.2 \times 10^6 \pm 1.6 \times 10^5$, $p < 0.05$, Figure 1A). The increases in macrophage count and lymphocyte count on day 7 were also attenuated in the montelukast group (macrophages: $6.3 \times 10^5 \pm 9.4 \times 10^4$ vs. $1.7 \times 10^5 \pm 3.2 \times 10^4$, $p < 0.05$, Figure 1B; lymphocytes: $8.6 \times 10^5 \pm 1.8 \times 10^5$ vs. 2.8 x

$10^5 \pm 8.1 \times 10^4$, $p < 0.05$, Figure 1C). However, there were no significant differences between the two groups, in the number of macrophages or lymphocytes on day 14 or in the number of other type of cells on day 7 or day 14.

Histological analysis

Histological evaluation of hematoxylin and eosin stained sections in the bleomycin group revealed extensive accumulation of many inflammatory cells, thickening of alveolar walls and fibrotic lesions on day 14 day after the bleomycin injection. By contrast, a reduction in the inflammatory and fibrotic changes in the subpleural areas of the lung was observed in the montelukast group (Figure 2). Histological analysis by the Ashcroft score showed a lower degree of pulmonary fibrosis in the montelukast group than in the bleomycin group on day 14 after the bleomycin injection ($n = 10$, $p < 0.01$, Table 1).

Hydroxyproline

The hydroxyproline content of the lung was lower in the motelukast group than in the bleomycin group ($n = 6$, $p < 0.05$, Table 1).

CysLTs level in BALF

The cysLTs level in the BALF was not significantly different between the motelukast group and the bleomycin group on day 14, although the level showed a lower tendency in the montelukast group ($n = 7$, $p = 0.170$, Table 1).

Assessment of TGFβ

TGFβ was evaluated by immunohistochemical staining of lung sections on day 14 after the bleomycin injection. Stronger TGFβ expression was detected in the macrophages and

pulmonary and bronchial epithelial cells of the lung sections in bleomycin group than in the montelukast group (Figure 3), and the TGFβ level in the BALF was significantly lower in the montelukast group than in the bleomycin group ($n = 6$, $p < 0.05$, Table 1).

Discussion

The results of this study show that montelukast significantly attenuated pulmonary fibrosis and reduced the TGFβ level in BALF and TGFβ expression in the lung tissue of the bleomycin-treated mice.

Recent evidence suggests that LTs play a pivotal role in pulmonary fibrosis. For example, much higher levels of LTs are present in lung homogenates from IPF patients than from healthy controls (Wilborn et al., 1996), and the LT level in BALF increases in bleomycin-treated mice, and the cysLTs levels, in particular, are much higher than the levels of the other eicosanoids (Nagase et al., 2002). Furthermore, the increase in inflammatory cells in BALF and lung tissues of bleomycin-treated 5-LO gene knockout mice and cytosolic phospholipase A2 gene knockout mice are much lower than in wild-type mice, suggesting cysLTs and CysLT receptors may be involved in the inflammatory process of bleomycin-induced pulmonary fibrosis. Our cell analysis data demonstrated lower numbers of both macrophages and lymphocytes in BALF in the montelukast group on day 7. Macrophages produce various enzymes, cytokines, chemokines and inflammatory mediators, including cysLTs. Since CysLT1 receptors are expressed in monocytes/macrophages and fibroblasts in the lungs of mice (Kanaoka and Boyce, 2004) and cysLTs induce monocyte chemoattractant protein 1 production in macrophages/monocytes (Ichiyama et al., 2005), montelukast probably inhibits macrophage-mediated inflammation by the suppression of CysLT1 receptors on macrophages. Further study is needed to clarify this mechanism.

The decrease in Ashcroft score and lung hydroxyproline content and reduced TGFβ level in BALF and reduced TGFβ expression in lung sections detected immunohistochemically in this study indicated that montelukast attenuated the development of fibrosis. TGFβ is well known to be a critical growth factor in the fibrotic stage of pulmonary fibrosis and to play an important role in its pathogenesis, including bleomycin-induced fibrosis (Giri et al., 1993). Macrophages and epithelial cells immunostained positive for TGFβ in our study, suggesting that these cells are the main source of TGFβ production. LTC4 has been found to induce TGFβ production, which is inhibited by motelukast in airway epithelial cells (Perng et al., 2006), and inflammatory mediators and TGFβ, which are elevated in pulmonary fibrosis, amplify LT synthesis (Steinhilber et al., 1993). Therefore, montelukast may interrupt the vicious cycle of LT synthesis and TGFβ production, and as a result attenuate the fibrotic process.

CysLT1 receptor antagonists, such as montelukast, have been widely used in the treatment of asthma, and their efficacy in asthma is well established. Montelukast is reported not only to reduce but to reverse airway remodeling, including subepithelial collagen deposition, in a mice model of asthma (Henderson et al., 2002, 2006). Although our data also demonstrated that montelukast is effective against pulmonary fibrosis, Beller et al. reported that targeted disruption of the CysLT1 receptor significantly increased the magnitude of septal thickening in the lungs of bleomycin-treated mice (Beller et al., 2004). The precise reason for the discrepancy between their findings and ours is uncertain, but it may have been attributable to the difference between the genetic effect and acute effect of inhibitors. One possible mechanism may be associated with the level of cysLTs production. Beller’s data showed that the cysLTs level in the BALF was 2.5-fold higher in the CysLT1 receptor knockout mice than in the wild-type mice, whereas our data showed that the cysLTs level in

the BALF was not significantly different between the motelukast group and the bleomycin group.

IPF is a progressive, fatal disease, and the death is often triggered by an acute exacerbation (Martinez, 2006). However, no effective therapy has ever been established. As far as we know, there have been no clinical reports of pulmonary interstitial pneumonia and fibrosis as a side effect of montelukast used to treat asthma. Although our data are limited to the acute effect of montelukast on bleomycin-induced pulmonary fibrosis, montelukast may become a new therapy for IPF, including for the treatment of acute exacerbations.

In summary, our study demonstrated that montelukast inhibits the development of bleomycin-induced pulmonary fibrosis in mice.

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Figure legends

Figure 1

Cell analysis of bronchoalveolar fluid on day 7 and day 14 after bleomycin injection.

A) Total cell count. The increase in total cell count in bronchoalveolar lavage on day 7 and day 14 was attenuated in the montelukast group.

B) Differential macrophage cell count. The increase in macrophage count in bronchoalveolar lavage on day 7 was attenuated in the montelukast group.

C) Differential lymphocyte cell count. The increase in lymphocyte count in bronchoalveolar lavage on day 7 was attenuated in the montelukast group.

Data are reported as means ± SE. n = 6. * $p < 0.05$ vs. non-treated group. # $p < 0.05$ vs. bleomycin group.

Figure 2

Representative lung sections on day 14 after bleomycin injection.

H&E stain. Original magnification x100.

A) Bleomycin group

B) Montelukast group

Figure 3

Representative lung sections immunohistochemically stained for TGFβ on day 14 after the bleomycin injection. Original magnification x200.

A) Bleomycin group

B) Montelukast group

Table 1 The effect of montelukast in bleomycin-induced pulmonary fibrosis on day 14

Mouse group	Ashcroft score	Hydroxyproline (μg/right lung)	BALF cysLTs (pg/ml)	BALF TGFβ (pg/ml)
Non-treated group	0.0 ± 0.0	389.3 ± 24.6	2.6 ± 1.7	34.7 ± 5.7
Bleomycin group	2.8 ± 0.3P**	649.2 ± 61.4*	361.2 ± 148.9*	156.5 ± 27.3**
Montelukast group	1.8 ± 0.2$^{**\ \#\#}$	422.3 ± 42.6$^{\#}$	175.0 ± 57.5	87.0 ± 20.5$^{\#}$

Data are shown mean ± SE. n = 6-10. * $p < 0.05$, ** $p < 0.01$ vs. non-treated group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. bleomycin group.

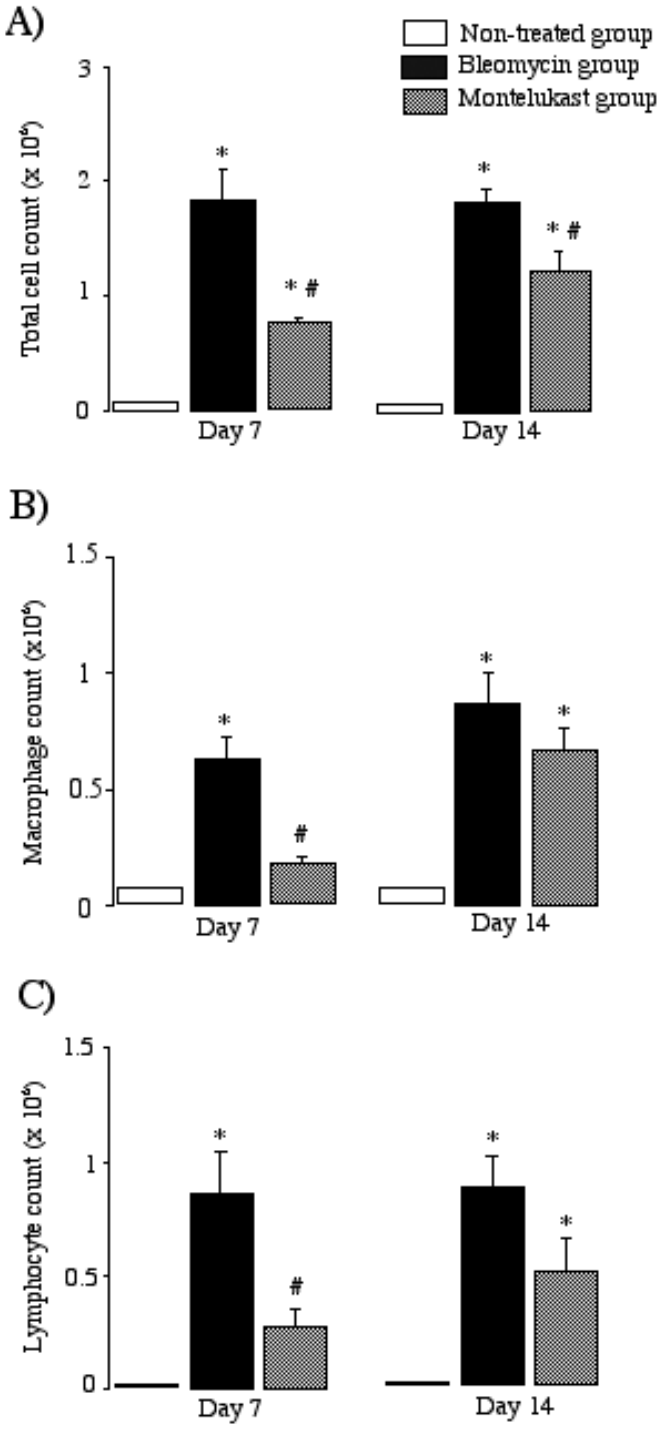
A)
Non-treated group
Bleomycin group
Montelukast group
Total cell count (x 10⁴)
3
2
1
0
*
* #
*
* #
Day 7
Day 14
B)
Macrophage count (x10⁴)
1.5
1
0.5
0
*
#
*
*
Day 7
Day 14
C)
Lymphocyte count (x 10⁴)
1.5
1
0.5
0
*
#
*
*
Day 7
Day 14

Fig.1

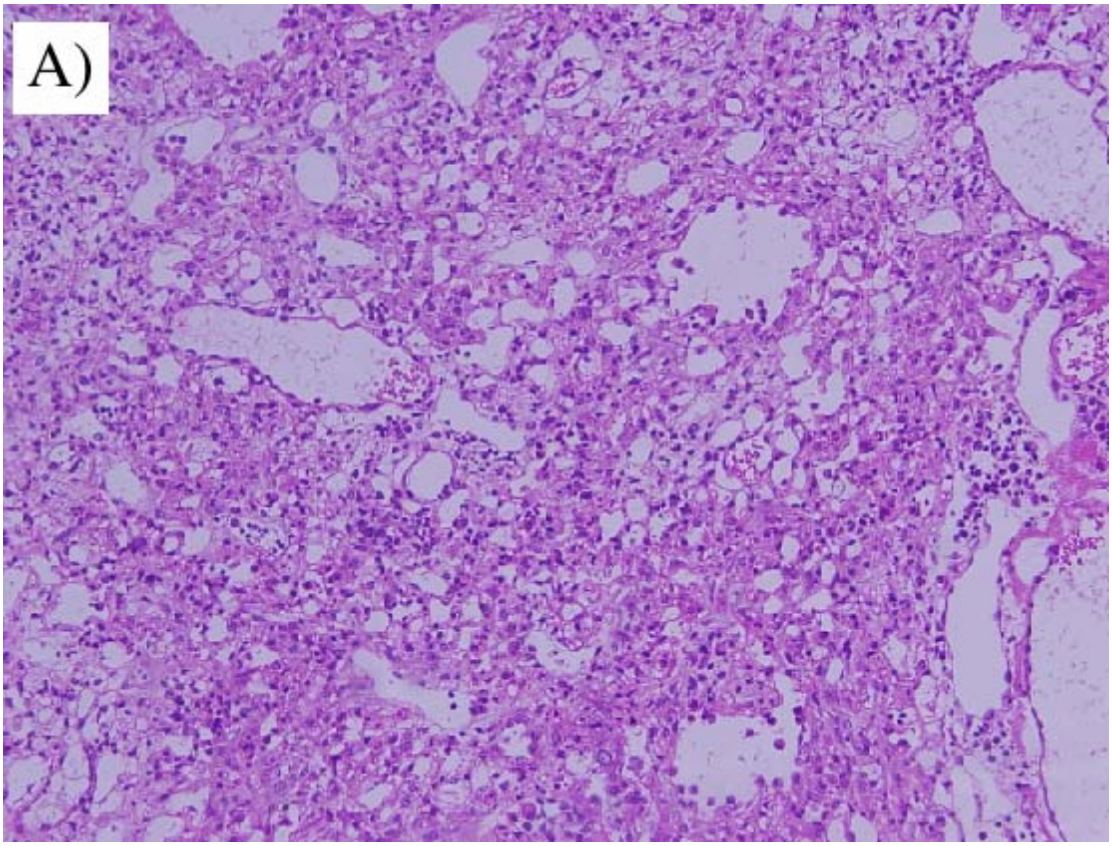

Fig.2A

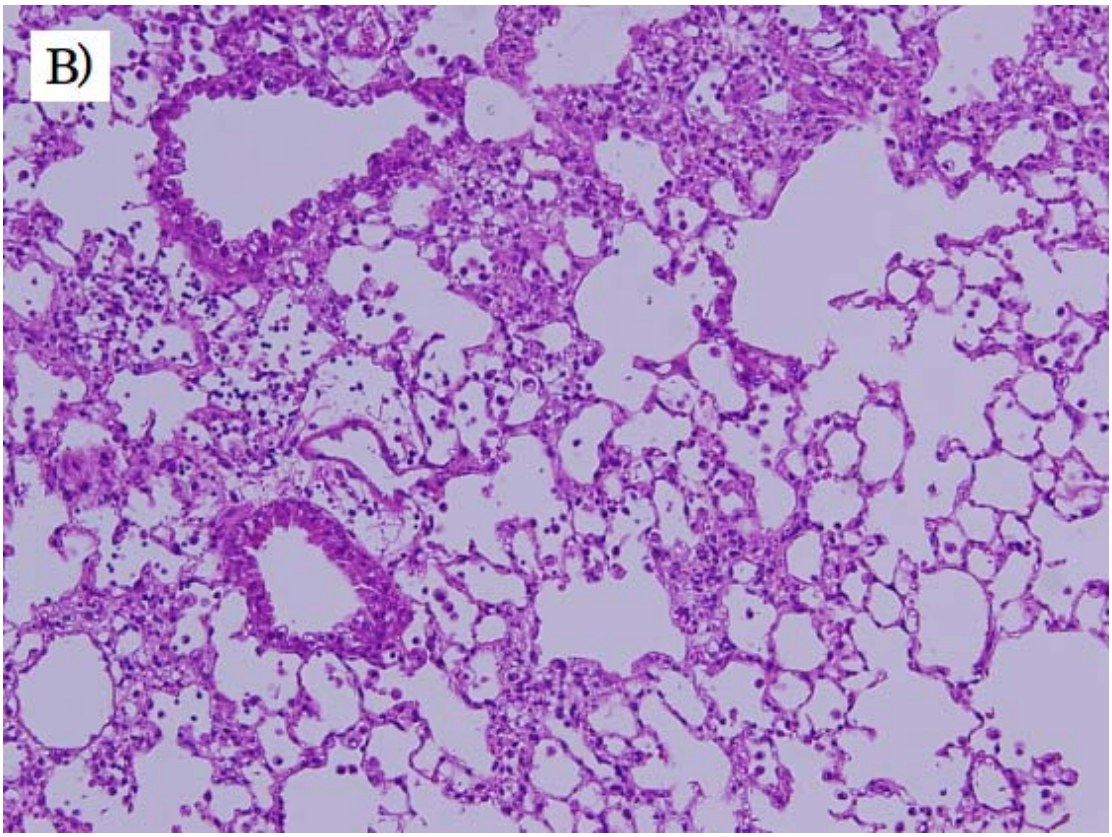

Fig.2B

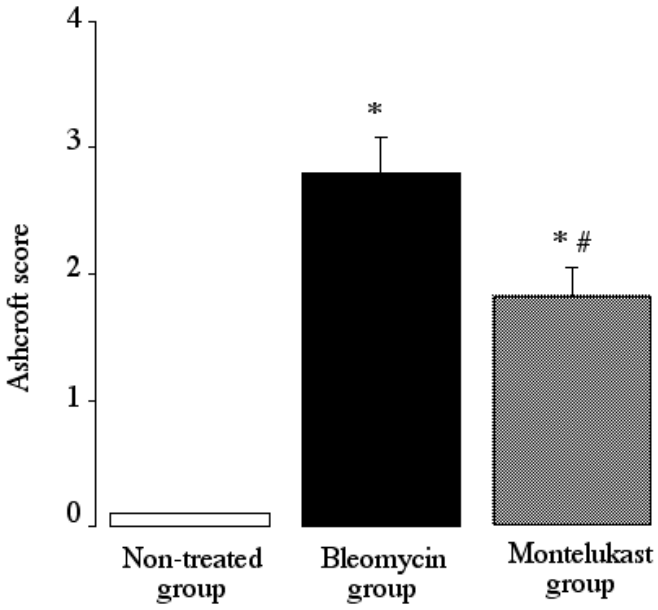
4
3
2
1
0
Ashcroft score
*
* #
Non-treated group
Bleomycin group
Montelukast group

Fig.3

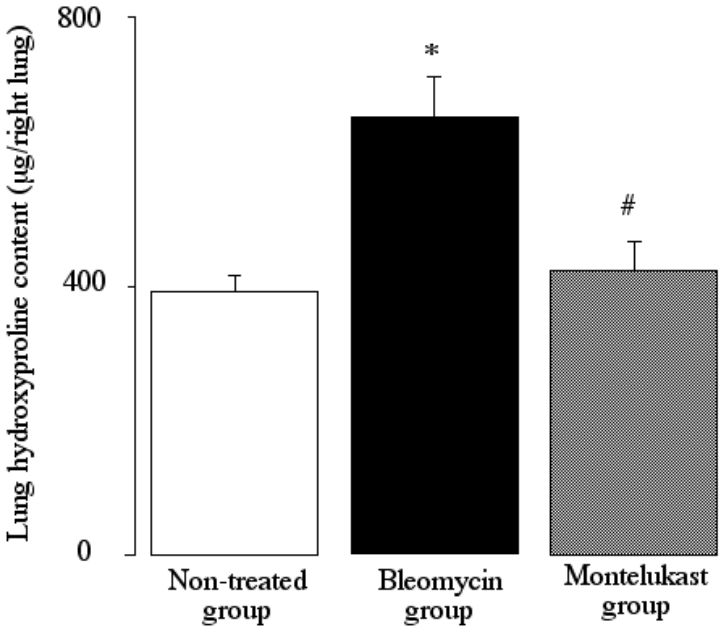
Lung hydroxyproline content (μg/right lung)
800
400
0
*
#
Non-treated group
Bleomycin group
Montelukast group

Fig.4

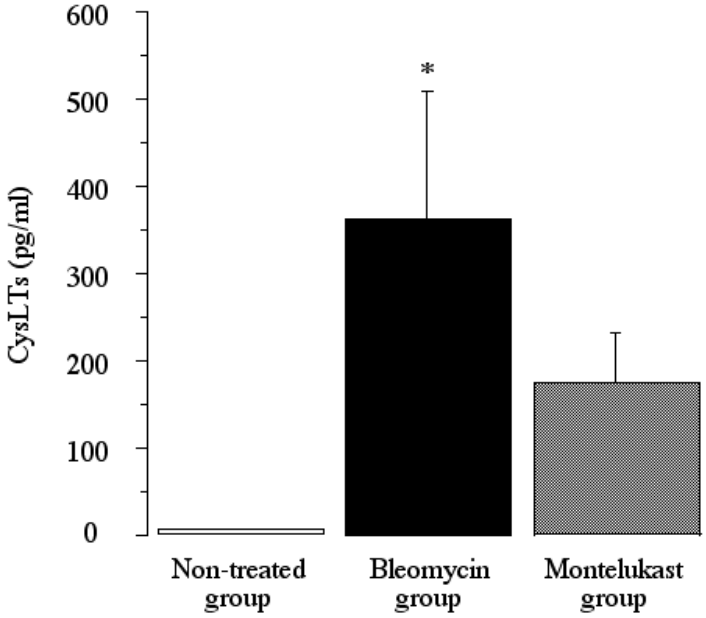
600
500
400
300
200
100
0
CysLTs (pg/ml)
*
Non-treated group
Bleomycin group
Montelukast group

Fig.5

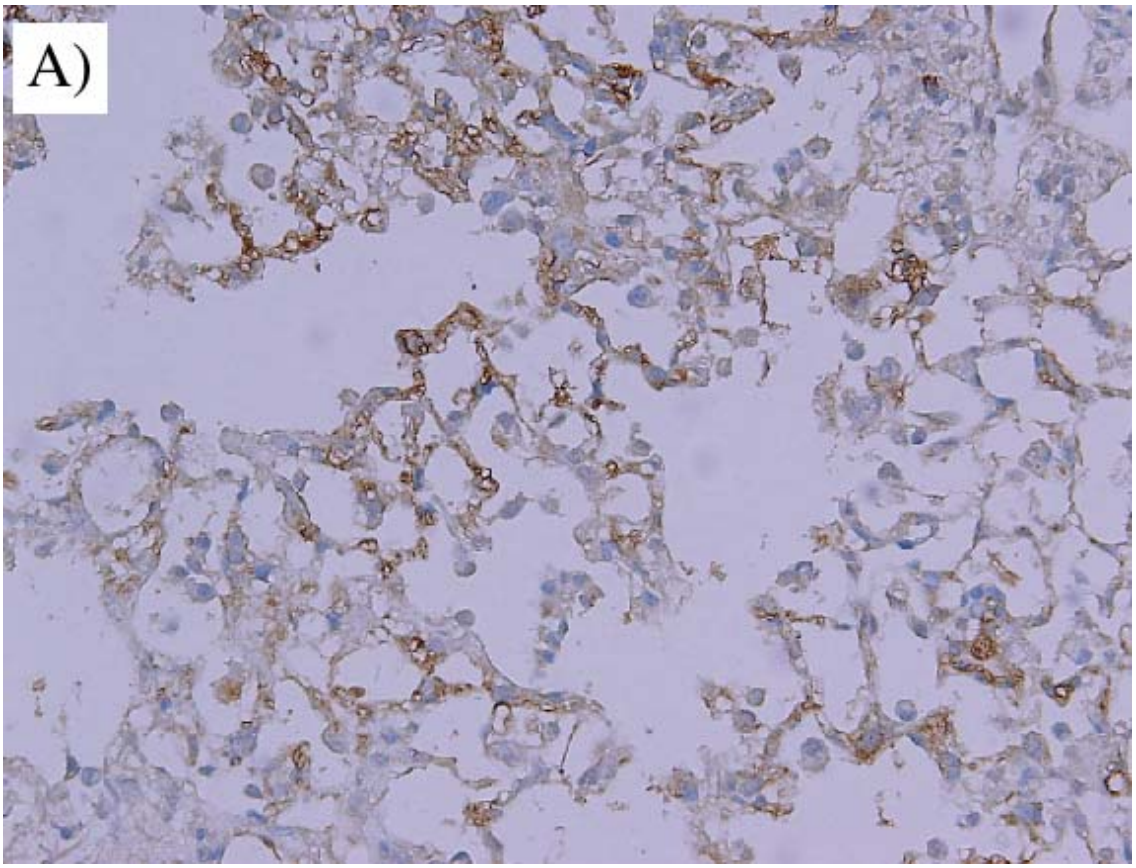

Fig.6A

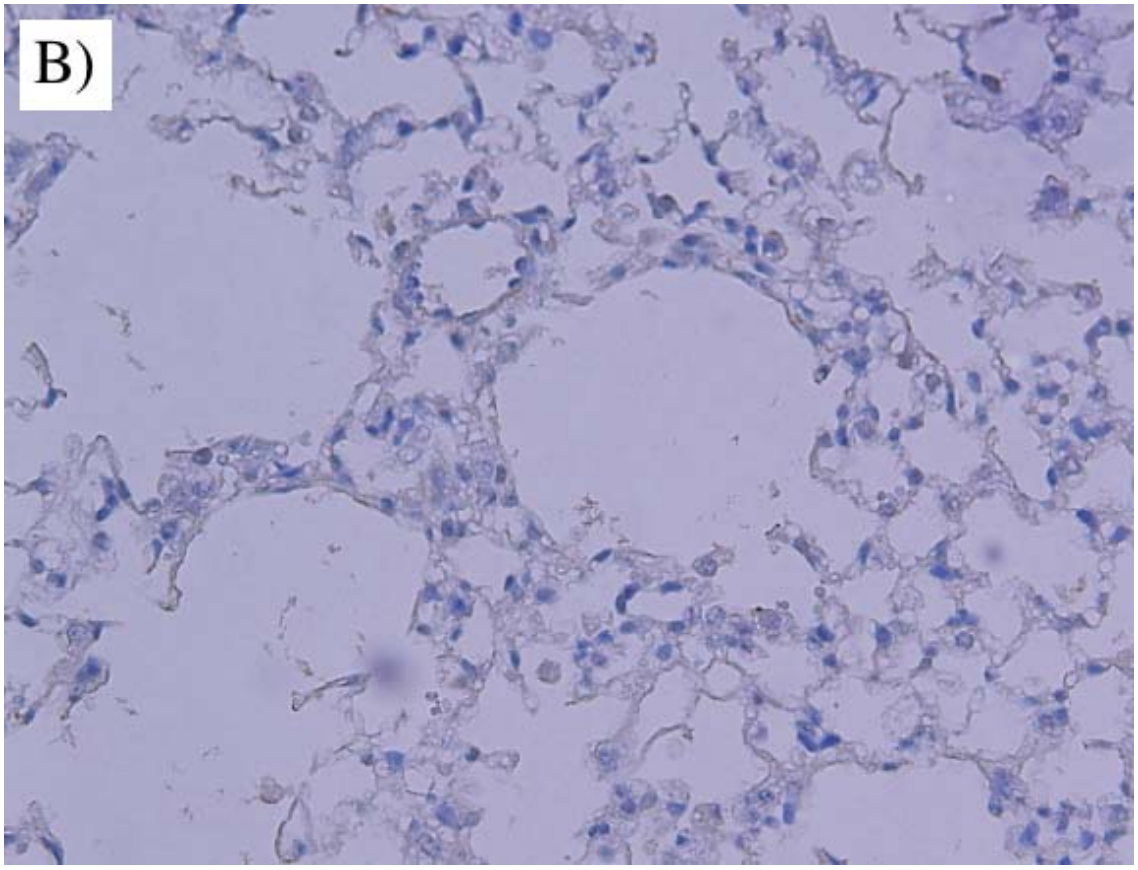

Fig.6B

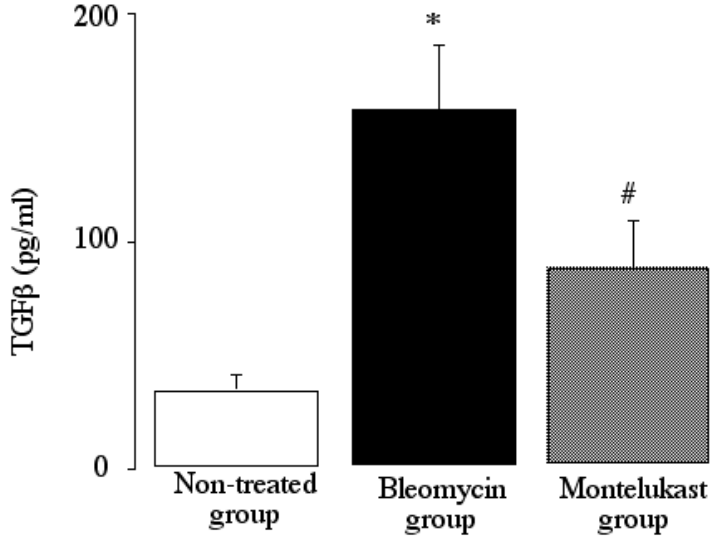
200
100
0
TGFβ (pg/ml)
*
#
Non-treated group
Bleomycin group
Montelukast group

Fig.7