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Effects of a leukotriene B_4 receptor antagonist on bleomycin-induced pulmonary fibrosis

Short title: BLTR ANTAGONIST INHIBITS PULMONARY FIBROSIS

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a devastating disease with poor prognosis. Leukotrienes play an important role in IPF, and leukotriene B_4 (LTB_4) is one of the key eicosanoids in IPF. In this study we investigated whether ONO-4057, a LTB_4 receptor (BLTR) antagonist is capable of preventing bleomycin-induced pulmonary fibrosis.

On day 1, C57BL/6 male mice were given a single intratracheal injection of bleomycin (2.5 mg/kg), and ONO-4057 (1.0 mg/kg) or vehicle alone was administered by intraperitoneal injection on days 1-5 each week for three weeks after the bleomycin injection.

ONO-4057 reduced the total cell count in bronchoalveolar lavage fluid (BALF) on days 7, 14, and 21 and the Ashcroft score and the lung hydroxyproline content on days 14 and 21. The LTB_4, interleukin-6 (IL-6), interleukin-13 (IL-13), transforming growth factor β (TGF-β) levels in BALF and TGF-β expression in lung tissue by immunohistochemistry were decreased on day 7, whereas interferon-γ (IFN-γ) level in BALF was increased on day 14.

The results of this study indicated that the BLTR antagonist inhibited the development of bleomycin-induced pulmonary fibrosis in mice by decreasing in the inflammation and altering TGF-β, IL-6, IL-13 and IFN-γ.

KEY WORDS

Bleomycin, interferon-γ, interleukin-6, leukotriene B_4, pulmonary fibrosis, transforming growth factor β

SHORT TITLE

BLTR ANTAGONIST INHIBITS PULMONARY FIBROSIS

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a deleterious disease with very poor prognosis despite all known methods of treatment [1]. The pathological features of IPF are fibroblast proliferation, increased amounts of extracellular matrix, and varying degrees of persistent inflammation of the alveolar septa [2]. Recent reports have suggested that leukotrienes (LTs), which are arachidonic acid metabolites, are important regulators of pulmonary fibrosis [3, 4]. The synthesis of leukotriene B_4 (LTB_4) and cysteinyl leukotriene (cysLT) is catalyzed by 5-lipoxygenase. LTB_4 plays an important role in the host defense system against infection and invasion by foreign bodies [5]. LTB_4 is thought to be a cause of various inflammatory disorders, and it is produced by alveolar cells in patients with IPF [6]. In fact, LTB_4 level is elevated in bronchoalveolar lavage fluid and lung tissues in patients with IPF [7, 8]. There are two LTB_4 receptor (BLTR) subtypes, BLT1 and BLT2, and both subtypes are G-protein-coupled receptors and present on the cell surface [9]. BLT1 is primarily expressed in leukocytes, whereas BLT2 is expressed more ubiquitously. There have been several reports concerning the role of BLTR in the

airway disease. According to one report, BLT1 mediates LTB_4-induced T helper type 1 (Th1) and Th2 cell chemotaxis and firm adhesion to endothelial cells exposed to flow, and mediates $CD4^+$ and $CD8^+$ T cell recruitment into the airway in an asthma model [10]. BLT1-mediated T cell trafficking is critical to the development of rejection and obliterative bronchiolitis after lung transplantation by mediating airway fibroproliferation [11]. However, the role of BLTR in pulmonary fibrosis has remained unclear. We recently reported finding that montelukast, one of the cysLT1 receptor antagonists that have been widely used in the treatment of bronchial asthma, inhibits the development of bleomycin-induced pulmonary fibrosis [12].

In the present study, we attempted to determine whether ONO-4057, a BLTR antagonist, has any preventive effect on bleomycin-induced pulmonary fibrosis in mice. ONO-4057 (5-[2-(2-carboxyethyl)-3-{6-(4-methoxyphenyl)-5E-hexenyl} oxyphenoxy] valeric acid) (ONO Pharmaceutical, Osaka, Japan) is a non-selective BLTR antagonist that predominantly antagonizes BLT1, and has been reported to inhibit human neutrophil aggregation, chemotaxis and degranuation induced by LTB_4 [9, 13]. We also focused on the effects of ONO-4057 on PGE_2, active TGF-β1, IL-4, IL-6, IL-13 and IFN-γ levels in

BALF and on TGF-β expression in lung tissue in pulmonary fibrosis, because these mediators have been found to be strongly associated with the pathogenesis of pulmonary fibrosis [14-16].

MATERIALS AND METHODS

Animals and bleomycin-induced pulmonary fibrosis model

The animal protocol was approved by the Animal Care and Use Committee of Tokyo Women's Medical University. We performed the study in 6-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. ONO-4057 (1.0 mg/kg) was supplied by Ono Pharmaceutical Co. (Osaka, Japan), and the animals were intraperitoneally injected with ONO-4057 or with vehicle (0.14% sodium bicarbonate) alone on days 1-5 of each week for three weeks starting two hours after the bleomycin injection, or on days 15-19 after bleomycin injection. Thus, there were four groups of mice in this study: a saline-injected group treated with the vehicle (vehicle-treated group, n = 10), a bleomycin-injected group treated with the vehicle (bleomycin group, n = 10), a bleomycin-injected group treated with ONO-4057 starting two hours after the intratracheal injection of bleomycin (ONO-4057 group, n = 10), and a bleomycin-injected group treated with ONO-4057 starting 15 days after the intratracheal injection of bleomycin (ONO-4057 delayed initiation group, n = 10). We evaluated the

severity of the lung inflammation by performing a bronchoalveolar lavage fluid (BALF) analysis on days 7, 14 and 21, and we evaluated the effect of ONO-4057 on pulmonary fibrosis by histological evaluation by means of the Ashcroft score [17] and based on the hydroxyproline content of the right lung on days 14 and 21 [18].

BALF analysis

After the anesthetizing mice with pentobarbital (50 mg/kg, ip), a tracheotomy was performed, and a custom-made cannula was inserted. The lungs were lavaged with 1.0 ml of PBS and then with 0.8 ml of PBS. The BALF was centrifuged at 360 *g* for 10 min, and the supernatant was stored at -80˚C for the subsequent measurements. 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 a differential count of 1000 cells per sample was made. Eicosanoid and cytokine levels in BALF were measured on days 7 and 14 by using an enzyme-linked immunosorbent assay (ELISA). The LTB_4 level in BALF was measured by using ELISA (Amersham Biosciences, Piscataway, NJ), and the detection limit of the assay was 6 pg/ml. The PGE_2 level in BALF was measured with

a mouse PGE_2 ELISA kit (GE Healthcare, Buckinghamshire, UK), and the detection limit was 50 pg/ml. The IL-4, IL-6, IL-13, IFN-γ, and active TGF-β1 levels in BALF were measured with a mouse IL-4 ELISA kit, a mouse IL-6 ELISA kit, a mouse IL-13 ELISA kit, a mouse IFN-γ ELISA kit, and a mouse TGF-β1 ELISA kit (R&D system, Minneapolis, MN), and the detection limit were 2.0 pg/ml, 1.6 pg/ml, 1.5 pg/ml, 2.0 pg/ml and 4.2 pg/ml, respectively.

Histological analysis

The left lung was fixed by inflation with 4% paraformaldehyde and embedded in paraffin (n = 10). Sections were cut 5 μm thick, and stained with hematoxylin and eosin. The Ashcroft score was used for semiquantitative analysis of fibrotic change on day 14 and on day 21 as reported previously [17].

Immunostaining for TGF-β

For immunostaining of TGF-β, after deparaffinizing in sections xylene and dehydrating in them 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 TGF-β antibody (diluted 1:50; Santa Cruz Biotechnology, Inc. San Diego, CA), and control sections were incubated at 4˚C overnight with a rabbit immunoglobulin fraction as a negative control (diluted 1:1000; DakoCytomation). The following day all sections were washed three times with PBS. Antibody that had bound to TGF-β 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 [18]. In brief, lung tissues were hydrolyzed with 12 N hydrochloric acid at 110˚C for 24 hours. After neutralization with sodium hydroxide, the hydrolysates were diluted with distilled water. The absorbance at 560 nm was measured.

Statistical analysis

Data are reported as means ± SEM. Statistical analysis was ANOVA followed by Sheffe's F test as a post hoc analysis test. Differences with P values of less than 0.05 were considered statistically significant.

RESULTS

Cell analysis of BALF

The total cell count in BALF was markedly higher in the bleomycin group than in the vehicle-treated group, and the count on days 7, 14 and 21 showed that the increase was significantly inhibited by ONO-4057 ($n = 10$, $p < 0.01$, fig. 1a). The increase in neutrophil count on day 7 was attenuated significantly in the ONO-4057 group ($n = 10$, $p < 0.01$, fig. 1b). The increase in macrophage count on day 14 was attenuated in the ONO-4057 group ($n = 10$, $p < 0.01$, fig. 1c). The increase in lymphocyte count was attenuated in the ONO-4057 group on day 14 and on day 21 and in the ONO-4057 delayed initiation group on day 21 ($n = 10$, $p < 0.05$, $p < 0.01$, $p < 0.01$, respectively, fig. 1d).

Histological analysis

Histological evaluation of hematoxylin and eosin stained sections in the bleomycin group revealed extensive accumulation of numerous inflammatory cells, thickening of alveolar walls, and fibrotic lesions on day 21 after the bleomycin injection (fig. 2a). By contrast, less severe inflammatory and fibrotic changes in the subpleural areas of the lung were

observed in the ONO-4057 group (fig. 2b). Histological analysis by the Ashcroft score showed a lower degree of pulmonary fibrosis in the ONO-4057 group than in the bleomycin group on days 14 and 21 after the bleomycin injection ($n = 10$, $p < 0.01$, $p < 0.01$, respectively, fig. 3a). But the ONO-4057 delayed initiation group failed to attenuate the lung fibrosis by the Ashcroft score on day 21 (fig. 2c, 3a).

Hydroxyproline

The hydroxyproline content of the right lung on day 14 and on day 21 was lower in the ONO-4057 group than in the bleomycin group (day 14; bleomycin group vs. ONO-4057 group; 522.5 ± 31.9 μg/right lung vs. 383.4 ± 32.5 μg/right lung, $n = 10$, $p < 0.05$, day 21; bleomycin group vs. ONO-4057 group; 601.3 ± 31.8 μg/right lung vs. 430.5 ± 40.9 μg/right lung, $n = 10$, $p < 0.01$, fig. 3b). However, the ONO-4057 delayed initiation group on day 21 did not result in a significant reduction in the hydroxyproline (584.1 ± 34.8 μg/right lung, $n = 10$, fig. 3b).

Eicosanoid levels in BALF

On day 7, the LTB_4 level in the BALF was significantly higher in the bleomycin group than the vehicle-treated group (vehicle-treated group vs. bleomycin group; 35.9 ± 10.2 pg/ml vs. 192.1 ± 56.4 pg/ml, n = 10, $p < 0.05$, fig. 4a), and was lower in the ONO-4057 group than in the bleomycin group (bleomycin group vs. ONO-4057 group; 192.1 ± 56.4 pg/ml vs. 59.7 ± 26.3 pg/ml, n = 10, $p < 0.05$, fig. 4a). Although the LTB_4 level tended to be higher in the bleomycin group than that in the vehicle-treated group on day 14 (n = 10, $p = 0.053$, fig. 4a), there were no significant differences between the bleomycin group and the ONO-4057 group on day 14 (bleomycin group vs. ONO-4057 group; 160.2 ± 61.1 pg/ml vs. 97.1 ± 38.0 pg/ml, n = 10, $p = 0.21$, fig. 4a). PGE_2 levels were increased after bleomycin injection, but there were no significant differences in PGE_2 level between the bleomycin group and the ONO-4057 group on day 7 and on day 14 (day 7; bleomycin group vs. ONO-4057 group; 3506.8 ± 873.9 pg/ml vs. 3713.0 ± 824.0 pg/ml, n = 10, $p = 0.999$, day 14; bleomycin group vs. ONO-4057 group; 4431.8 ± 729.7 pg/ml vs. 3780.9 ± 912.0 pg/ml, n = 10, $p = 0.985$, fig. 4b).

Cytokine levels in BALF

There were no significant differences between the IL-4 level in BALF in each group (bleomycin group vs. ONO-4057 group, day 7: 14.6 ± 1.2 pg/ml vs. 11.5 ± 1.3 pg/ml, day 14: 11.3 ± 0.9 pg/ml vs. 13.6 ± 0.8 pg/ml n = 10, fig. 5a). On day 7, the IL-6 level in BALF was lower in the ONO-4057 group than in the bleomycin group (bleomycin group vs. ONO-4057 group, 1003.9 ± 151.3 pg/ml vs. 508.2 ± 135.0 pg/ml, n = 10, $p < 0.01$, fig. 5b). On day 7, the IL-13 level in BALF was lower in the ONO-4057 group than in the bleomycin group (bleomycin group vs. ONO-4057 group, 24.0 ± 2.3 pg/ml vs. 17.4 ± 1.1 pg/ml, n = 10, $p < 0.05$, fig. 5c), but there were no significant differences in the IL-13 level between the bleomycin group and the ONO-4057 group on day 14 (bleomycin group vs. ONO-4057 group, 20.2 ± 1.2 pg/ml vs. 22.6 ± 1.2 pg/ml, n = 10, fig. 5c), On day 14, the IFN-γ level in BALF was higher in the ONO-4057 group than in the bleomycin group (bleomycin group vs. ONO-4057 group, 11.8 ± 5.2 pg/ml vs. 53.8 ± 17.3 pg/ml, n = 10, $p < 0.05$, fig. 5d), but the IFN-γ level in BALF on day 7 tended to be higher, but not significant different (bleomycin group vs. ONO-4057 group, 27.6 ± 11.5 pg/ml vs. 51.2 ± 16.1 pg/ml, n = 10, $p = 0.14$, fig. 5d).

Assessment of TGF-β

TGF-β was evaluated by immunohistochemical staining of lung sections on day 7 after the bleomycin injection. Stronger TGF-β expression was detected in the neutrophils and pulmonary epithelial cells of the lung sections in the bleomycin group than in the ONO-4057 group (fig. 6a and 6b), and active TGF-β1 level in BALF was increased on day 7 and on day 14 in the bleomycin group, and ONO-4057 inhibited active TGF-β1 level on day 7 (bleomycin group vs. ONO-4057 group: 204.5 ± 24.3 pg/ml vs. 98.1 ± 11.4 pg/ml, $p < 0.01$, $n = 10$, fig. 6c). However, there were no significant differences in active TGF-β1 level between the bleomycin group and the ONO-4057 group on day 14 ($n = 10$, fig. 6c).

DISCUSSION

The results of our study show that ONO-4057 attenuated pulmonary inflammation and fibrosis, reduced IL-6, IL-13, active TGF-β1 level and increased IFN-γ level in BALF, and attenuated TGF-β expression in the lung tissue of bleomycin-treated mice.

LTs are important regulators of pulmonary fibrosis [3,4]. LTB_4 and cysLT are increased in BALF and lung homogenates of patients with IPF, and the levels of these mediators were found to correlate with the extent of fibrosis in histological sections [7, 8]. In the human IPF lung tissue, the content of LTB_4 is greater than that of cysLT. By contrast, Peters-Golden et al. reported that the cysLT level greatly exceeded the LTB_4 level in the BALF in bleomycin-induced mouse model [19]. Furthermore Failla et al reported the efficacy of pharmacological inhibition of LTs activity in the development of bleomycin-induced lung injury with mice treated with zileuton, a 5-lipoxygenase inhibitor and MK-571, a cysLT1 receptor antagonist. As the potency of zileuton for the inhibition of bleomycin-induced injury was similar to that of MK-571, they speculated that LTB_4 dose not have a predominant role in mediating inflammation and fibrosis in their bleomycin-treated mice [20]. However, our results showed that the LTB_4 level was

significantly higher on day 7 and tended to be higher on day 14 in the bleomycin-treated mice than in the vehicle-treated mice, and the level was comparable to the cysLT level as shown in our previous report [12]. Furthermore ONO-4057, a BLT inhibitor decreased the LTB_4 level in BALF on day 7 and ameliorated bleomycin-induced pulmonary inflammation and fibrosis. These results suggest that LTB_4 as well as cysLT plays an important role in bleomycin-induced pulmonary fibrosis in our mouse model. The reason of the discrepancy between others and ours is uncertain, but the difference in mouse strain, bleomycin dose, and the pharmacological mechanism of action (a synthetic enzyme inhibitor or a receptor antagonist) may have been influenced.

On the other hand, the PGE_2 level between the bleomycin group and the ONO-4057 group was not different, suggesting that the effects of ONO-4057 are unrelated to prostaglandin production. Thus, ONO-4057 did not affect the cycloxygenase pathway. This result was similar to the previous report that nordihydroquiaretic acid (NDGA), 5-lipoxygenase inhibitor did not affect macrophage-derived PGE_2 production in bleomycin-induced pulmonary fibrosis [21].

ONO-4057 is a specific LTB_4 receptor antagonist that inhibits the human neutrophil aggregation, chemotaxis, and degranulation induced by LTB_4 [13]. In our study, the neutrophil number and the LTB_4 level in BALF on day 7 was decreased by ONO-4057, indicating that neutrophil chemotaxis, aggregation and degranulation were suppressed. On the other hand, LTB_4 plays an important role in antimicrobial lung defense through its production by alveolar macrophages and neutrophils, and the use of BLT1 antagonist may compromise host immune response to pulmonary infection [3], although ONO-4057 (1 mg/kg) did not decrease the neutrophil number and the LTB_4 level below the levels in the vehicle-treated group on day 7. Further examination may be necessary for clinical application of BLT1 antagonist.

In the present study, LTB_4 and active TGF-β1 levels in BALF were increased on day 7 and on day 14 in the bleomycin group, but were inhibited by ONO- 4057 only on day 7. As neutrophils were accumulated on day 7, the source of TGF-β on day 7 may have been derived from neutrophils [22]. In fact, neutrophils and epithelial cells on day 7 immunostained positive for TGF-β in our study, suggesting that these cells are the main source of TGF-β production. By contrast, the source of LTB_4 and TGF-β on day 14 may

have been derived from macrophages that were predominant cells on day 14. Although ONO-4057 failed to inhibit LTB_4 and active TGF-β1 levels on day 14 in BALF, ONO-4057 inhibited macrophage number on day 14. In view of the fact that BLTRs are expressed in the spleen and on leukocytes [23], ONO-4057 probably inhibits neutrophil- and macrophage-mediated inflammation by suppressing of BLTRs on neutrophils and macrophages, and as a result inhibited TGF-β production especially by neutrophils. TGF-β 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 in the pathogenesis of bleomycin-induced fibrosis. Since neutralization of TGF-β with antibodies mitigates bleomycin-induced pulmonary fibrosis, and exogenous over-expression of Smad7, an inhibitor of the TGF-β signaling pathway, also mitigates bleomycin-induced pulmonary fibrosis, inhibition of the effects of TGF-β may provide an effective means of treating pulmonary fibrosis [24].

IFN-γ level in BALF was increased by ONO-4057 on day 14. IFN-γ has several effects that may play an important role at the interface between inflammation and fibrosis in bleomycin-induced pulmonary fibrosis. IFN-γ has been found to inhibit collagen

synthesis when cultures of fibroblasts have been exposed to them [25]. IFN-γ eliminates the upregulation of collagen synthesis induced by TGF-β [26]. Thus, it suggests that the increase in IFN-γ as well as the decrease in TGF-β by ONO-4057 may induce the inhibition of collagen synthesis.

Several recent papers have suggested that LTB_4 functions not only as a local inflammatory mediator, but also as an important chemoattractant for T cells as well as neutrophils [10]. In an asthma model, BLT1 mediates LTB_4-induced Th1 and Th2 cell chemotaxis and firm adhesion to endothelial cells exposed to flow, and mediates $CD4^+$ and $CD8^+$ T cell recruitment into the airway. In the present study, the number of lymphocyte was inhibited by ONO-4057 on day 14 and on day 21. Although the role of lymphocytes in the pathogenesis of the pulmonary fibrotic response has not been fully clarified, several studies have emphasized the importance and diverse roles of these cells in the fibrotic process [10]. There are two major subsets of T-helper lymphocytes. One subset, Th1 cells, produces IL-2 and IFN-γ, and mediates cellular immune responses. The other subset, Th2 cells, produces IL-4, IL-5, IL-6 and IL-13 etc. and augments humoral immune responses. Th1/Th2 imbalance is proposed to be one of the hypotheses related to

developing pulmonary fibrosis. ONO-4057 reduced IL-13 level but not IL-4 on day 7 and increased IFN-γ on day 14 (fig. 5). These results suggest that ONO-4057 may improve Th1/Th2 balance. However, compared with these cytokines, IL-6 level in BALF was extremely elevated on day 7 in our bleomycin-induced mouse model, and ONO-4057 clearly reduced IL-6. Several studies have demonstrated that IL-6 has both pro-inflammatory and anti-inflammatory properties [27]. IL-6 also exhibits a negative feedback on the process of fibrosing. In fact, the IL-6 level of BALF is greatly increased in patients with IPF and in the bleomycin-induced model of pulmonary fibrosis [27]. High IL-6 levels are correlated with alveolar hypercellularity and neutrophil counts in IPF [28]. Recently, IL-6-deficient mice demonstrated attenuation in bleomycin-induced lung injury and fibrosis [29]. Therefore, one of the mechanisms for the inhibition of inflammation and fibrosis by ONO-4057 may be associated with the decrease in IL-6.

In the present study, ONO-4057 delayed initiation group decreased the lymphocyte inflammation on day 21, but failed to decrease the hydroxyproline and the Ashcroft score on day 21. These results suggest that the prevention of developing fibrosis by ONO-4057 may be associated with inhibiting the subsequent fibrosis after

inflammation rather than fibrotic process *per se*. However, as a period of ONO-4057 administration in ONO-4057 delayed initiation group was short (on days 15 to 19), the possibility cannot be denied that longer administration of ONO-4057 may attenuate the development of fibrosis thereafter. Further examination would be needed.

In conclusion, ONO-4057 inhibited inflammation by the decrease in the inflammatory cells including neutrophils, macrophages and lymphocytes. More importantly, the decrease in Ashcroft score and lung hydroxyproline content, the decrease in active TGF-β1 level in BALF, and the reduced TGF-β expression detected immunohistochemically in the lung sections indicated that ONO-4057 mitigated the development of fibrosis. Current therapy for pulmonary fibrosis is ineffective, and the disease has a poor outcome and is associated with severe morbidity [30]. The BLTR antagonist shows promise of becoming a new means of treating IPF.

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FIGURE LEGENDS

FIGURE 1.

Effects of ONO-4057 on cell counts of bronchoalveolar fluid on day 7, 14 and 21 after the bleomycin injection.

a) Total cell count. The increase in total cell count in bronchoalveolar lavage fluid on day 7, day 14 and day 21 was attenuated in the ONO-4057 group, and also was attenuated on day 21 in the ONO-4057 delayed initiation group.

b) Differential neutrophil cell count. The increase in neutrophil count in bronchoalveolar lavage fluid on day 7 was attenuated in the ONO-4057 group.

c) Differential macrophage cell count. The increase in macrophage count in bronchoalveolar lavage fluid on day 14 was attenuated in the ONO-4057 group.

d) Differential lymphocyte cell count. The increase in lymphocyte count in bronchoalveolar lavage fluid on day 14 and day 21 was attenuated in the ONO-4057 group, and also was attenuated on day 21 in the ONO-4057 delayed initiation group.

Data are reported as means ± SEM (n = 10 in each group).

Open bars: Vehicle-treated group; solid bars: Bleomycin group; shaded bars: ONO-4057 group, hatched bar: ONO-4057 delayed initiation group.

- $p < 0.05$ vs. Vehicle-treated group. ** $p < 0.01$ vs. Vehicle-treated group. # $p < 0.05$ vs. Bleomycin group. ## $p < 0.01$ vs. Bleomycin group.

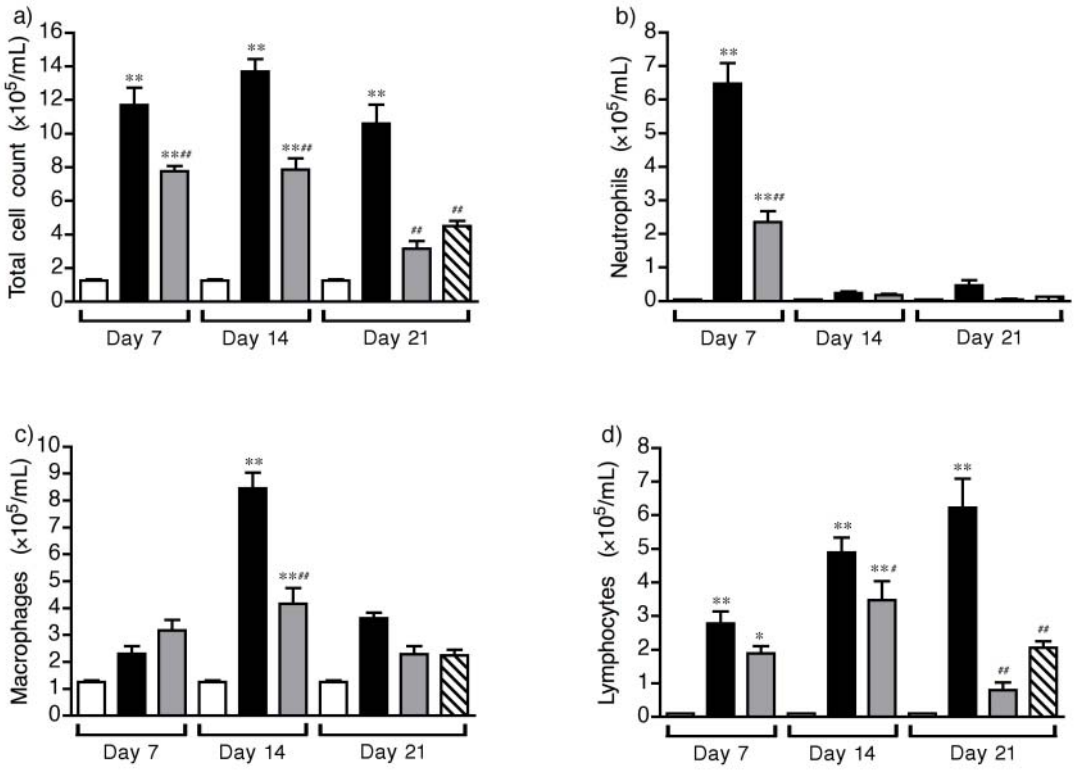


FIGURE 2.

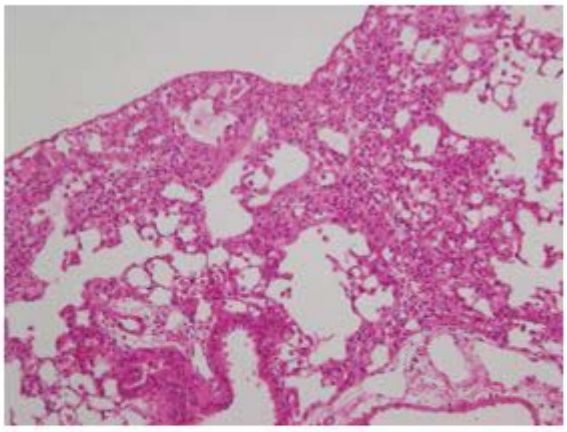
Effects of ONO-4057 on histopathological changes of the lung on day 21 after the bleomycin injection. Representative lung sections stained with H & E. Original magnification ×100.

a) A lung section from the bleomycin group showing widespread accumulation of numerous inflammatory cells, thickening of the alveolar walls, and fibrotic lesions on day 21 after the bleomycin injection.

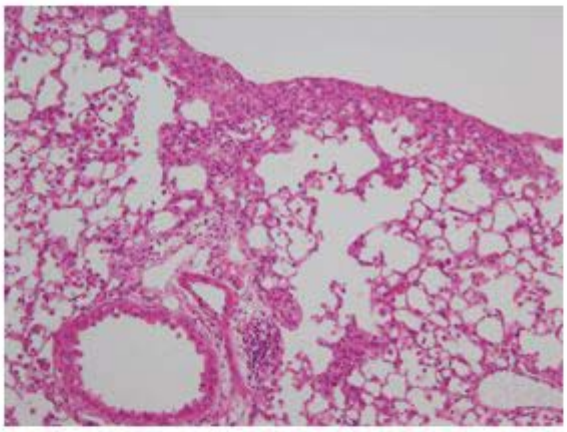
b) By contrast, less severe inflammatory and fibrotic changes in the subpleural areas of the lung were observed in the ONO-4057 group.

c) The histological changes in the ONO-4057 delayed initiation group were the middle grade of the bleomycin group and the ONO-4057 group.

a)



b)



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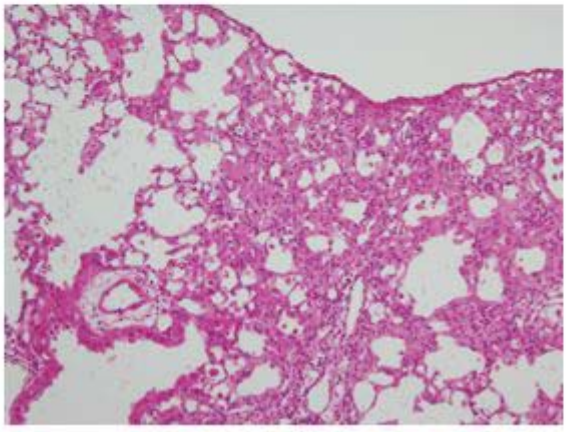


FIGURE 3.

Effects of ONO-4057 on the Ashcroft score and the lung hydroxyproline content of the right lung on day 14 and on day 21 after the bleomycin injection.

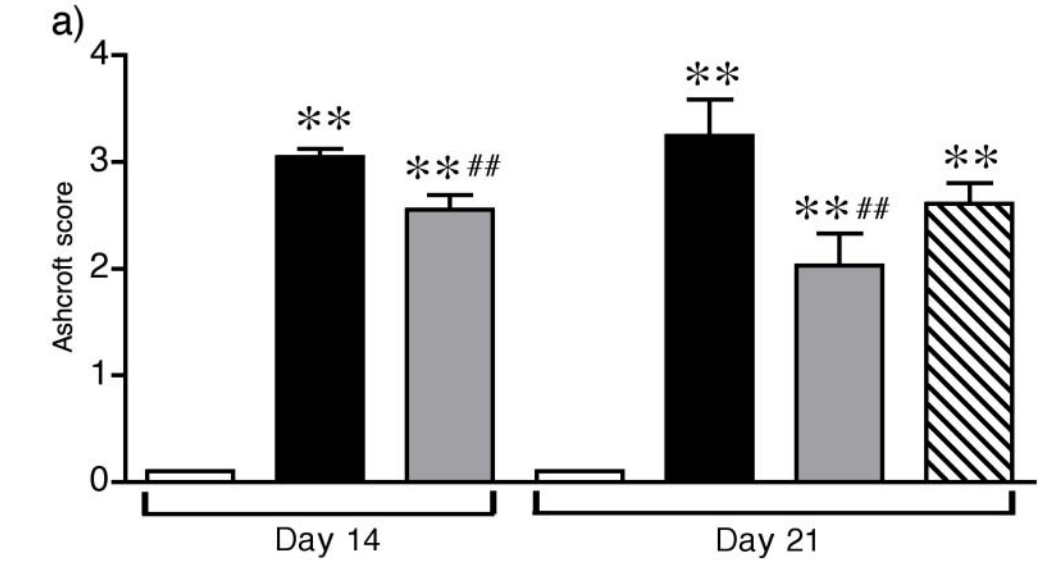
a) The Ashcroft score after the bleomycin injection showed a lower degree of pulmonary fibrosis in the ONO-4057 group than in the bleomycin group on day 14 and on day 21.

b) The hydroxyproline content of the right lung was lower in the ONO-4057 group than in the bleomycin group on day 14 and on day 21.

Data are reported as means ± SEM (n = 10 in each group).

Open bars: Vehicle-treated group; solid bars: Bleomycin group; shaded bars: ONO-4057 group, hatched bar: ONO-4057 delayed initiation group.

- $p < 0.05$ vs. Vehicle-treated group. ** $p < 0.01$ vs. Vehicle-treated group. # $p < 0.05$ vs. Bleomycin group. ## $p < 0.01$ vs. Bleomycin group.


a)
Ashcroft score
4
3
2
1
0
**
**##
**
**##
**
Day 14
Day 21

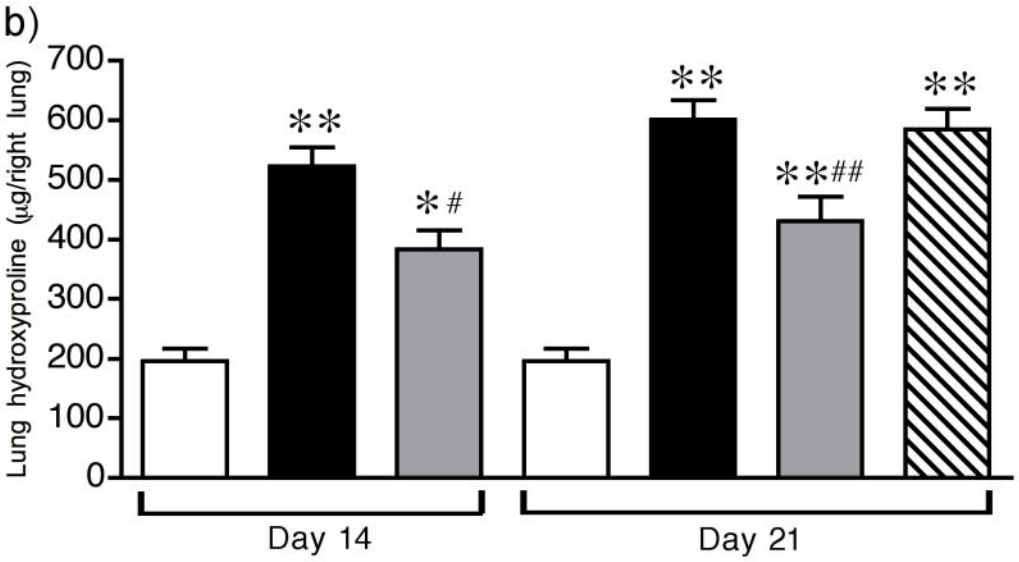

b)
Lung hydroxyproline (μg/right lung)
700
600
500
400
300
200
100
0
**
*#
**
**##
**
Day 14
Day 21

FIGURE 4.

Effects of ONO-4057 on the LTB_4 and PGE_2 level of bronchoalveolar fluid on day 7 and on day 14 after the bleomycin injection.

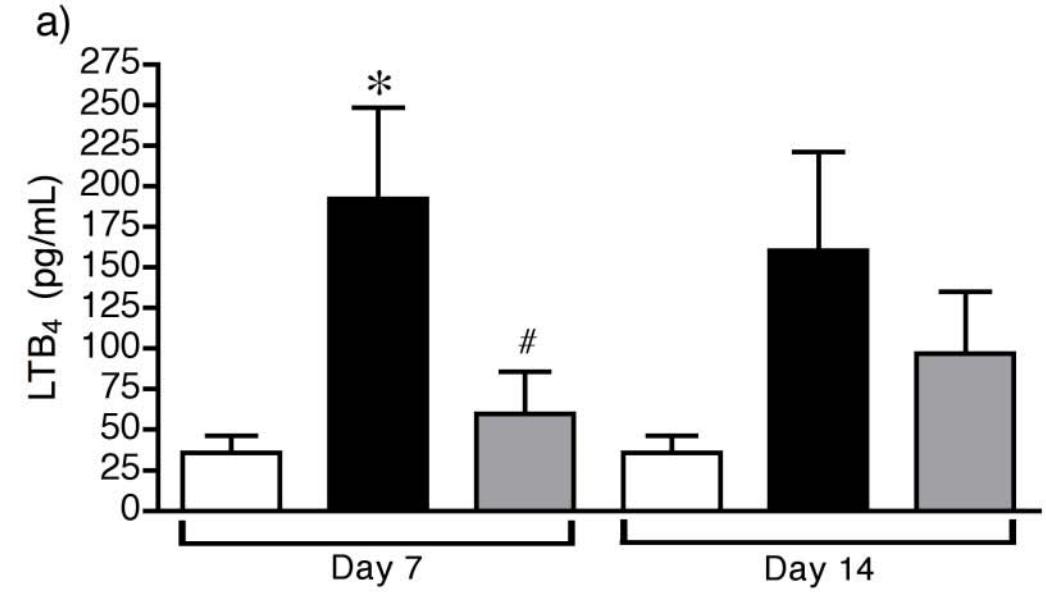
a) The LTB_4 levels were significantly higher in the bleomycin group than in the vehicle-treated group on day 7 ($p < 0.05$, n = 10), but not on day 14 ($p = 0.053$, n = 10). LTB4 level was lower in the ONO-4057 group than in the bleomycin group on day 7 ($p < 0.05$, n = 10), but not on day 14 ($p = 0.21$, n = 10).

b) There were no significant differences in PGE_2 level between the ONO-4057 group and the bleomycin group on day 7, and on day 14.

Data are reported as means ± SEM (n = 10).

Open bars: Vehicle-treated group; solid bars: Bleomycin group; shaded bars: ONO-4057 group.

- $p < 0.05$ vs. Vehicle-treated group. # $p < 0.05$ vs. Bleomycin group.


a)
LTB4 (pg/mL)
275
250
225
200
175
150
125
100
75
50
25
0
*
#
Day 7
Day 14

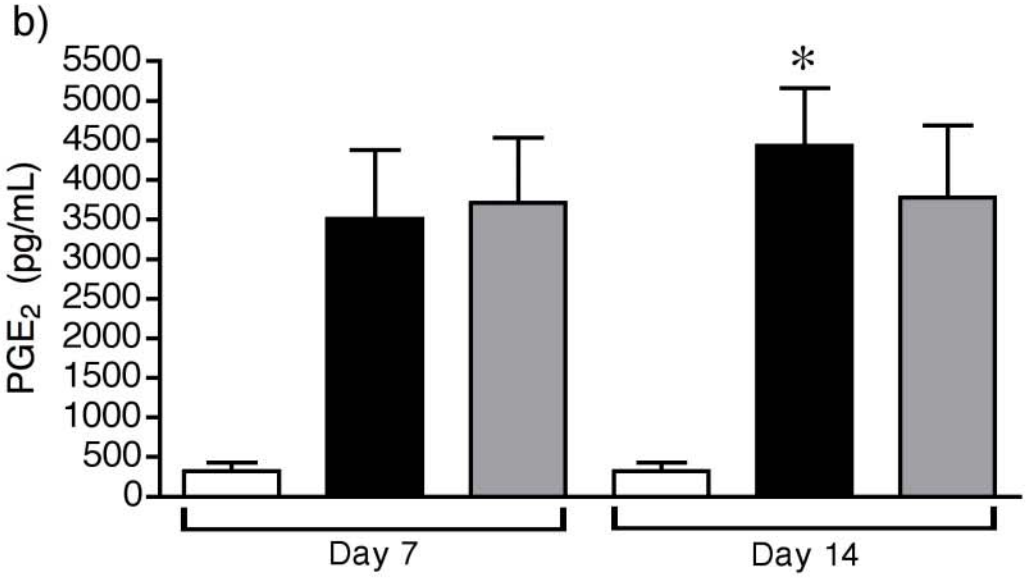

b)
PGE2 (pg/mL)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
Day 7
Day 14

FIGURE 5.

Effects of ONO-4057 on the IL-4, IL-6, IL-13 and IFN-γ levels of bronchoalveolar fluid on day 7 and on day 14 after the bleomycin injection.

a) There were no significant differences in the IL-4 level between each group on day 7 and on day 14.

b) The IL-6 level on day 7 was significantly reduced by ONO-4057.

c) The IL-13 level on day 7 was significantly reduced by ONO-4057.

c) The IFN-γ level on day 14 was significantly increased by ONO-4057.

Data are reported as means ± SEM (n = 10 in each group).

Open bars: Vehicle-treated group; solid bars: Bleomycin group; shaded bars: ONO-4057 group.

- $p < 0.05$ vs. Vehicle-treated group. # $p < 0.05$ vs. Bleomycin group. ## $p < 0.01$ vs. Bleomycin group.

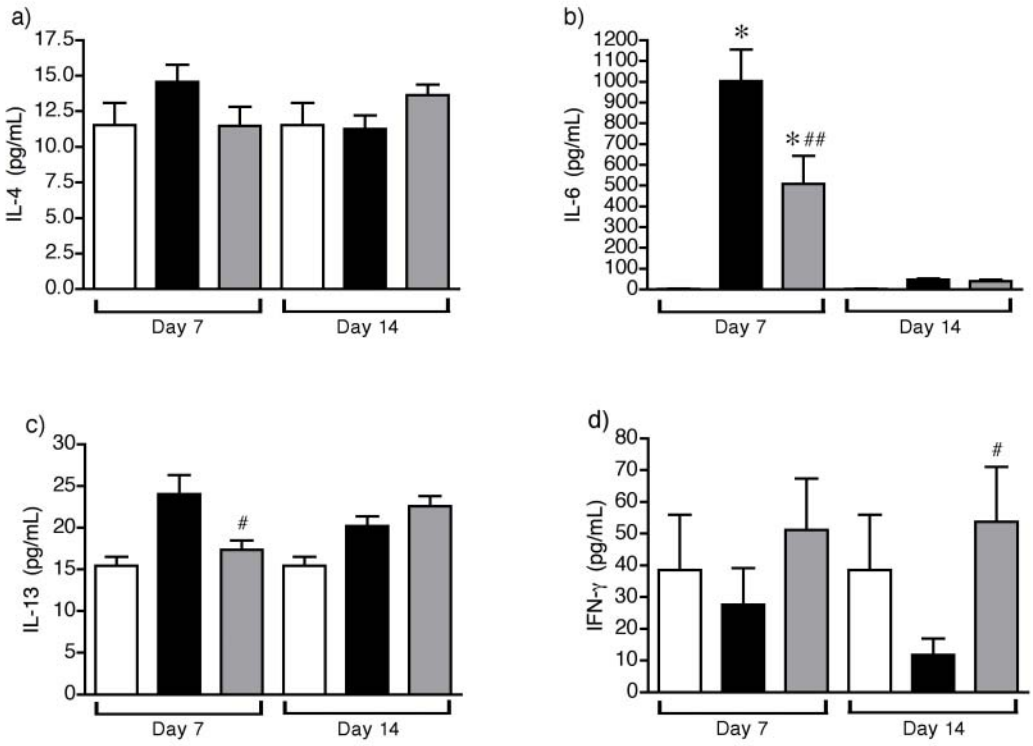


FIGURE 6.

Effects of ONO-4057 on immunohistochemically staining for TGF-β and on the active TGF-β1 level of bronchoalveolar fluid. Lung sections immunohistochemically stained for TGF-β on day 7 after the bleomycin injection. Original magnification ×400.

a) A lung section from the bleomycin group shows stronger TGF-β expression in the neutrophils and pulmonary epithelial cells.

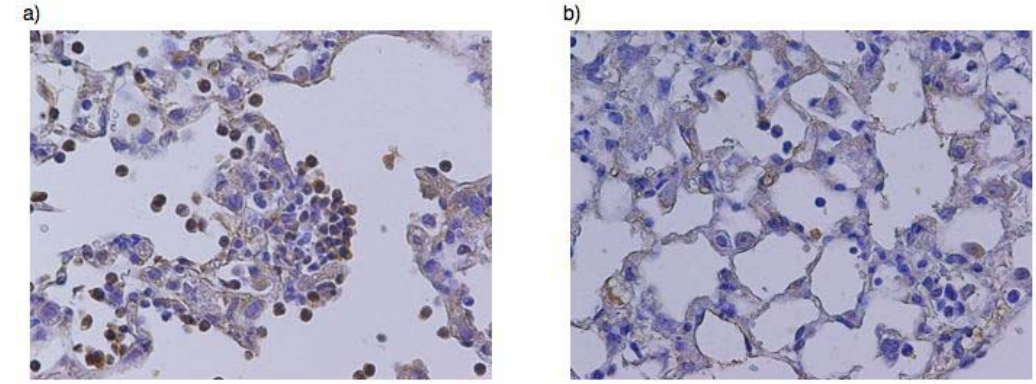
b) TGF-β expression was weaker in the ONO-4057 group.

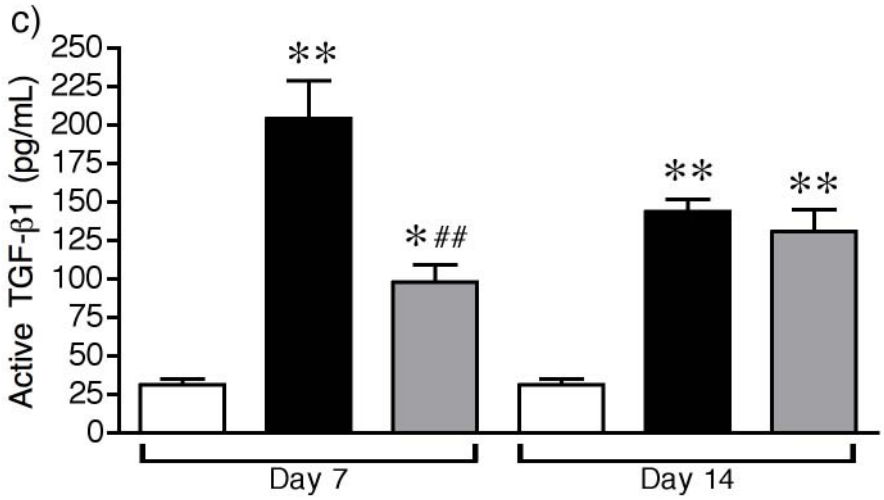
c) The active TGF-β1 level on day 7 was significantly lower in the ONO-4057 group than in the bleomycin group.

Data are reported as means ± SEM (n = 10 in each group).

Open bars: Vehicle-treated group; solid bars: Bleomycin group; shaded bars: ONO-4057 group.

- $p < 0.05$ vs. Vehicle-treated group. ** $p < 0.01$ vs. Vehicle-treated group. ## $p < 0.01$ vs. Bleomycin group.


a)
b)


c)
Active TGF-β1 (pg/mL)
250
225
200
175
150
125
100
75
50
25
0
**
*##
**
**
Day 7
Day 14