

## Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice

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Submitted 20 January 2005; accepted in final form 1 September 2005

**Murakami, Shinsuke, Noritoshi Nagaya, Takefumi Itoh, Masaharu Kataoka, Takashi Iwase, Takeshi Horio, Yoshinori Miyahara, Yoshiki Sakai, Kenji Kangawa, and Hiroshi Kimura.** Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 290: L59–L65, 2006. First published September 9, 2005; doi:10.1152/ajplung.00042.2005.—The balance between prostacyclin and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. We have recently developed ONO-1301, a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. Thus we investigated whether repeated administration of ONO-1301 attenuates bleomycin-induced pulmonary fibrosis in mice. After intratracheal injection of bleomycin or saline, mice were randomized to receive repeated subcutaneous administration of ONO-1301 or vehicle. Bronchoalveolar lavage (BAL) and histological analyses were performed at 3, 7, and 14 days after bleomycin injection. *In vitro* studies using mouse lung fibroblasts were also performed. ONO-1301 significantly attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by significant decreases in Ashcroft score and lung hydroxyproline content. ONO-1301 significantly reduced total cell count, neutrophil count, and total protein level in BAL fluid in association with a marked reduction of TXB<sub>2</sub>. A single administration of ONO-1301 significantly increased plasma cAMP level for >2 h. *In vitro*, ONO-1301 and a cAMP analog dose-dependently reduced cell proliferation in mouse lung fibroblasts. The reduction in cell proliferation by ONO-1301 was attenuated by a protein kinase A (PKA) inhibitor. Furthermore, bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle. These results suggest that repeated administration of ONO-1301 attenuates the development of bleomycin-induced pulmonary fibrosis and improves survival in bleomycin mice, at least in part by inhibition of TXA<sub>2</sub> synthesis and activation of the cAMP/PKA pathway.

adenosine 3',5'-cyclic monophosphate; fibroblast; neutrophil; survival

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a life-threatening disease characterized by progressive dyspnea and worsening of pulmonary function (6, 22). The pathological features of IPF are fibroblast proliferation with increased amounts of extracellular matrix and varying degrees of persistent inflammation of the alveolar septa (22). Thus a novel therapeutic strategy

against these abnormalities may be effective for the treatment of IPF.

Prostanoids, which are metabolites of arachidonic acid, are important regulators of pulmonary homeostasis. Prostacyclin inhibits migration, proliferation, and collagen synthesis in fibroblasts (14, 29). Conversely, thromboxane A<sub>2</sub> (TXA<sub>2</sub>) promotes fibroblast proliferation, increases pulmonary vascular permeability, and induces lung inflammation (18, 21, 24). Interestingly, a recent study has demonstrated that the decreased ratio of prostacyclin synthesis to thromboxane synthesis is associated with the development of pulmonary fibrosis (4). Thus we hypothesized that compounds that regulate the balance between prostacyclin and TXA<sub>2</sub> may have beneficial effects in IPF.

Recently, we have developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, named ONO-1301. Unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule, which contributes to the biological and chemical stability of this compound. As a result, this compound can be given by subcutaneous administration twice a day. Its inhibitory effect on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301 (30).

Thus the purpose of this study was to investigate whether modulation of prostacyclin/TXA<sub>2</sub> balance by ONO-1301 attenuates bleomycin-induced pulmonary fibrosis and improves survival in bleomycin-injected mice.

### METHODS

**Animals.** We used specific pathogen-free female C57BL/6 mice weighing 18–20 g. The mice were randomly given intratracheal injection of either bleomycin (Nippon Kayaku, Tokyo, Japan) or saline, and assigned to receive repeated administration of ONO-1301 or vehicle. This protocol resulted in the creation of three groups: sham mice given vehicle (Sham group, *n* = 34), bleomycin mice given vehicle (Vehicle group, *n* = 34), and bleomycin mice treated with ONO-1301 (ONO-1301 group, *n* = 34). Twenty-four additional mice were studied to evaluate the effect of ONO-1301 administration on survival in bleomycin mice. Twenty-five mice were studied to examine the effect of ONO-1301 on plasma cAMP. Finally, 15 mice were

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used to examine the effects of ONO-1301 on established pulmonary fibrosis. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute.

**ONO-1301.** We have recently developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, {7,8-dihydro-5-[(E)-2-[( $\alpha$ -(3-pyridyl)benzylidene)amino-oxy]ethyl]-1-naphthyl-oxy} acetic acid, named ONO-1301 (Fig. 1). This compound has two interesting structural features. First, as stated above, unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule. This structural feature contributes to the biological and chemical stability of this compound. Second, this compound possesses a 3-pyridine moiety at one end of the molecule and a carboxylic acid group at the other. The inhibitory effect of ONO-1301 on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301.

**In vivo experimental protocol.** After the mice were anesthetized by intraperitoneal injection of pentobarbital sodium, they were given intratracheal injection of either bleomycin (0.02 or 0.03 units/mouse) dissolved in 50  $\mu$ l of saline or saline alone. Then, ONO-1301 (6 mg $\cdot$ kg<sup>-1</sup> $\cdot$ day<sup>-1</sup>) dissolved in 100  $\mu$ l of saline, or saline was administered by subcutaneous injection twice a day throughout the experiment. This dose was determined to obtain maximum effects, based on dose-response experiments. Systolic blood pressure in the conscious state was measured by the indirect tail-cuff method with a blood pressure monitor (MK-2000; Muromachi Kikai, Tokyo, Japan) 0, 30, 60, 120, and 360 min after administration. ONO-1301 at a dose of 6 mg $\cdot$ kg<sup>-1</sup> $\cdot$ day<sup>-1</sup> did not influence blood pressure. The mice were maintained under standard conditions with free access to food and water.

The effects of ONO-1301 on pulmonary fibrosis were analyzed by the following parameters: 1) the severity of pulmonary fibrosis such as histological examination using the Ashcroft score and lung hydroxyproline content on day 14 (0.02 units/mouse), 2) the severity of acute lung injury such as that reflected in total cell count, differential cell count, and total protein level in bronchoalveolar lavage (BAL) fluid on day 3 and day 7 (0.02 units/mouse), 3) TXB<sub>2</sub> and active transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) levels in BAL fluid on day 3 and day 7 (0.02 units/mouse), 4) intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in whole lung tissue on days 3 and 7 (0.02 units/mouse), and 5) the survival rate on day 21 (0.03 units/mouse).

Finally, to investigate the effects of ONO-1301 on established pulmonary fibrosis, 15 mice were randomly given an intratracheal

injection of either bleomycin or saline. ONO-1301 or saline was administered from day 14 to 28 (Sham, Vehicle, and ONO-1301 groups,  $n = 5$  each). These mice were evaluated on day 28.

**BAL analysis.** Total and differential cell counts in BAL fluid were determined as described previously ( $n = 5$  each) (20). The supernatant of BAL fluid was used for the measurement of total protein, TXB<sub>2</sub>, which is a stable metabolite of TXA<sub>2</sub>, and active TGF- $\beta$ 1 levels. The total protein level was measured by Bradford assay (Bio-Rad, Tokyo, Japan). The TXB<sub>2</sub> level was measured with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The active TGF- $\beta$ 1 level was measured with a mouse TGF- $\beta$ 1 ELISA kit (R&D Systems, Minneapolis, MN).

**Histological examination.** The right lung was fixed by inflation with 4% paraformaldehyde and embedded in paraffin ( $n = 5$  each). Sections 4  $\mu$ m thick were stained with hematoxylin-eosin. The Ashcroft score was used for semiquantitative assessment of fibrotic changes (1). The severity of fibrotic changes in each histological section of the lung was assessed as the mean score of severity from observed microscopic fields. Thirty fields in each section were analyzed. Grading was performed in a blinded fashion by three observers, and the mean was taken as the fibrosis score.

**Measurement of hydroxyproline content.** To quantify lung collagen content as an indicator of pulmonary fibrosis, the hydroxyproline content in the lung was measured in each group according to the previously described method ( $n = 5$  each) (20). The left lung was quick-frozen and kept at -80°C until the assay. After the lung was homogenized, the suspension was hydrolyzed with 0.5 ml of 12 N hydrochloric acid for 20 h at 100°C. After neutralization, a 0.1 ml aliquot of supernatant was mixed in 1.5 ml of 0.3 N lithium hydroxide solution. The hydroxyproline content was analyzed by high-performance chromatography.

**Quantification of ICAM-1 and VCAM-1 expression by ELISA.** We investigated the effect of ONO-1301 on ICAM-1 and VCAM-1, which are key molecules in leukocyte migration into lung tissues, expression in the bleomycin-treated lung. The treated lungs were quick-frozen and kept at -80°C until the assay. The lungs were homogenized in 1.5 ml of saline. The homogenates were centrifuged at 2,000 g for 10 min at 4°C, and the supernatants were assayed for ICAM-1 and VCAM-1 concentrations by ELISA kits (R&D Systems).

**Measurement of cAMP level.** To evaluate the effect of ONO-1301 on plasma cAMP, normal mice were assigned to receive a single administration of ONO-1301 (3 mg/kg). Blood samples were obtained 0, 30, 60, 120, and 360 min after administration and were immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) and centrifuged immediately at 4°C

Fig. 1. Molecular structures of ONO-1301 and prostacyclin. Unlike prostacyclin, ONO-1301 does not possess a 5-membered ring and allylic alcohol, which contributes to the biological and chemical stability of this compound. ONO-1301 possesses a 3-pyridine moiety at 1 end of the molecule and a carboxylic acid group at the other, which contribute to inhibition of thromboxane synthesis. Green circle indicates prostacyclin activity; red circle indicates thromboxane synthase inhibitory activity; blue circle indicates 5-membered ring; orange circle indicates allylic alcohol.

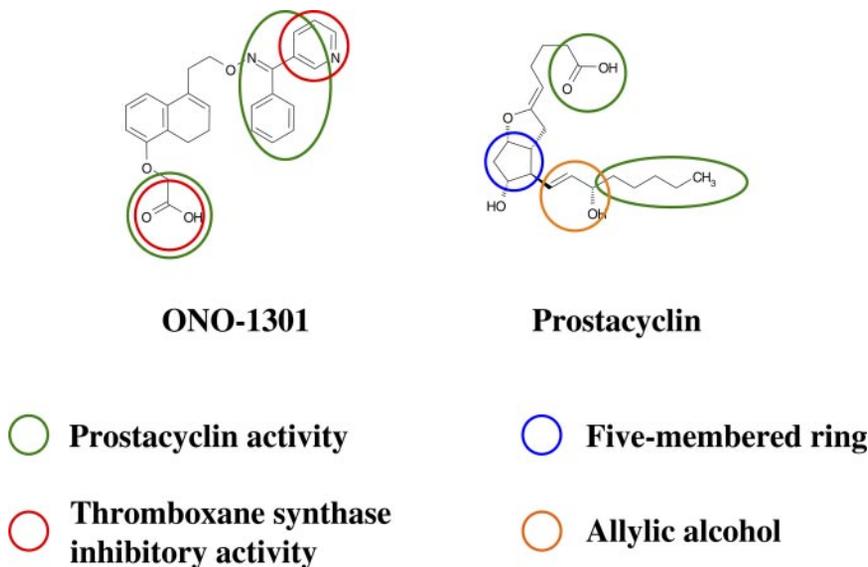


Table 1. *Physiological profiles of three experimental groups*

	Sham	Vehicle	ONO-1301
<i>n</i>	8	8	8
Body weight, g	22.1±0.2	19.3±0.6*	21.8±0.4†
Lung weight/body weight, mg/g	5.8±0.1	14.8±1.7*	9.4±0.5*†

Data are means ± SE. These measurements were performed at 14 days after bleomycin injection. Sham, sham mice given vehicle; Vehicle, bleomycin mice given vehicle; ONO-1301, bleomycin mice treated with ONO-1301; \**P* < 0.05 vs. Sham group; †*P* < 0.05 vs. Vehicle group.

(*n* = 5 each). The plasma cAMP level was measured with a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan) as described previously (13).

**Survival analysis.** To evaluate the effect of ONO-1301 on survival in bleomycin-injected mice, 24 mice received repeated administration of ONO-1301 (*n* = 12) or vehicle (*n* = 12) for 21 days. Survival was estimated from the date of bleomycin injection to the death of the mouse or 21 days after injection.

**In vitro study.** Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion with collagenase type III (10 mg/lung; Worthington Biochemical, Lakewood, NJ) for 80 min at 37°C, as reported previously (11). Cell suspension was filtered through 70-µm filters (BD Biosciences, Mountain View, CA). Then, these cells were centrifuged, washed, and cultured in complete medium composed of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Fibroblasts were used after the first cell passage. To evaluate the effect of ONO-1301 on intracellular cAMP, mouse lung fibroblasts grown in 24-well plates were incubated with various concentrations of ONO-1301 in the presence of 5 × 10<sup>-4</sup> M 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan), a phosphodiesterase inhibitor (*n* = 8 each), for 10 min. The intracellular cAMP level was measured with a radioimmunoassay kit as described previously (10). The effects of ONO-1301 and 8-bromo cAMP (Sigma, St. Louis, MO), a cAMP analog, on cell proliferation were assessed using a CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's directions (*n* = 8 each). Cells were treated for 48 h with fresh medium containing 2.5% FCS along with various concentrations of ONO-1301

or 8-bromo cAMP. The effect of ONO-1301 (10<sup>-6</sup> M) on cell proliferation in the presence of a myristoylated protein kinase A (PKA) inhibitor (10<sup>-6</sup> M) (Protein Kinase A Inhibitor 14–22 Amide, Cell-permeable, Myristoylated; Calbiochem, Cambridge, MA) was also evaluated. Finally, to investigate the underlying mechanism responsible for regulation of cell proliferation, mouse lung-derived fibroblasts were treated with various concentrations of imidazole (Wako Pure Chemical Industries, Osaka, Japan), a thromboxane synthesis inhibitor, or beraprost sodium (Cayman Chemical), a stable prostacyclin analog.

**Statistical analysis.** All data are expressed as means ± SE unless otherwise indicated. Comparisons were made by one-way ANOVA followed by Newman-Keuls test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of *P* < 0.05 was considered statistically significant.

**RESULTS**

**Physiological profiles.** The physiological profiles of the three experimental groups are shown in Table 1. Body weight was significantly lower in bleomycin mice given vehicle (Vehicle group) than in normal mice given vehicle (Sham group). However, a significant decrease in body weight was not observed in bleomycin mice treated with ONO-1301 (ONO-1301 group). Bleomycin injection significantly increased wet lung weight to body weight ratio. However, the increase was significantly attenuated by treatment with ONO-1301.

**Inhibition of pulmonary fibrosis.** The normal alveolar structure was maintained in the Sham group (Fig. 2A). Fourteen days after bleomycin injection, the alveolar walls were thickened and the air spaces were collapsed in the Vehicle group. In contrast to the findings in mice treated with bleomycin alone, pulmonary fibrosis was less severe in the ONO-1301 group. Semiquantitative assessment by the Ashcroft score demonstrated that the degree of pulmonary fibrosis in the ONO-1301 group was significantly lower than that in the Vehicle group (Fig. 2B). The hydroxyproline content in the lung was significantly increased after bleomycin injection (Fig. 2C). However,

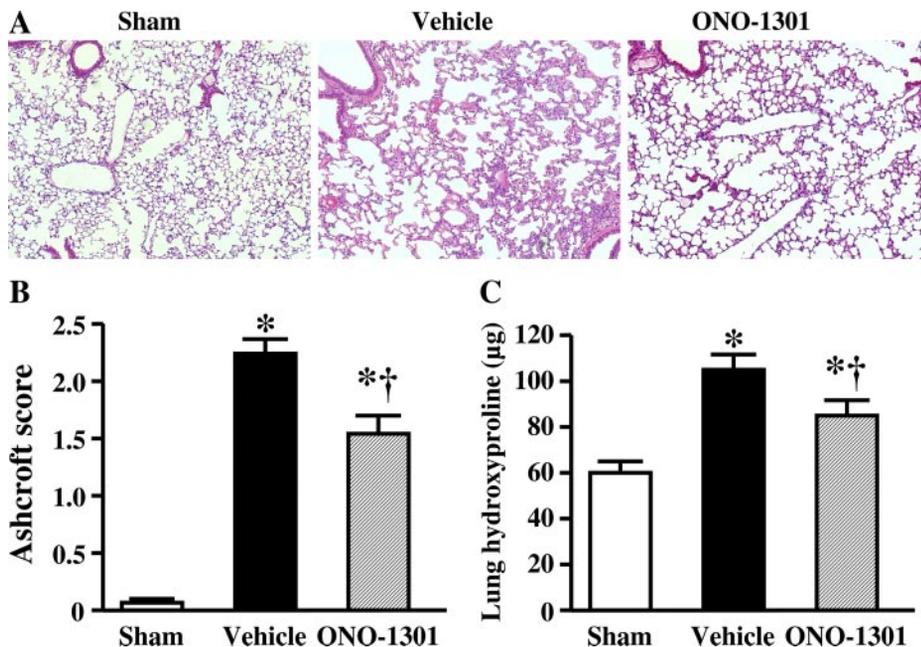
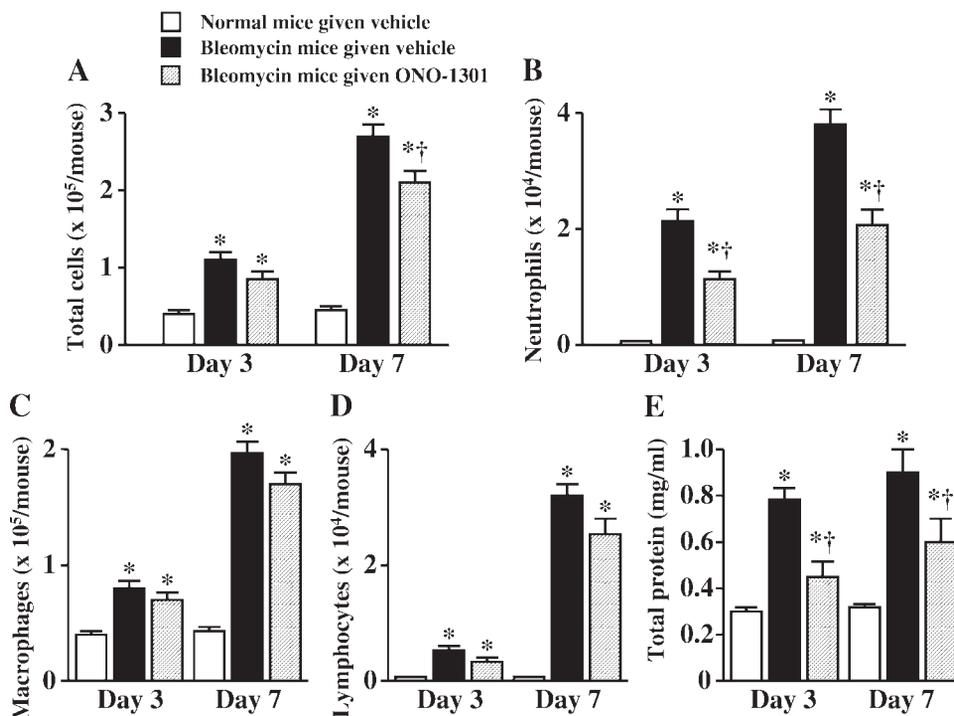


Fig. 2. A: representative photomicrographs of lung tissue at 14 days after bleomycin injection. Bleomycin-induced pulmonary fibrosis was attenuated by treatment with ONO-1301. Hematoxylin-eosin stain; magnification ×100. B: semiquantitative analyses of lung tissue using the Ashcroft score, a marker for pulmonary fibrosis. C: effect of ONO-1301 administration on hydroxyproline content in left lung of bleomycin-injected mice. Data are means ± SE. \**P* < 0.05 vs. Sham group; †*P* < 0.05 vs. Vehicle group.

Fig. 3. Effects of ONO-1301 administration on total and differential cell counts (macrophages, *C*; lymphocytes, *D*) in bronchoalveolar lavage (BAL) fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increases in total cell count at 7 days (*A*) and neutrophil count at 3 and 7 days (*B*) after bleomycin injection. *E*: effect of ONO-1301 administration on total protein level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increase in total protein level in BAL fluid. Data are means  $\pm$  SE. \* $P < 0.05$  vs. Sham group, † $P < 0.05$  vs. Vehicle group.



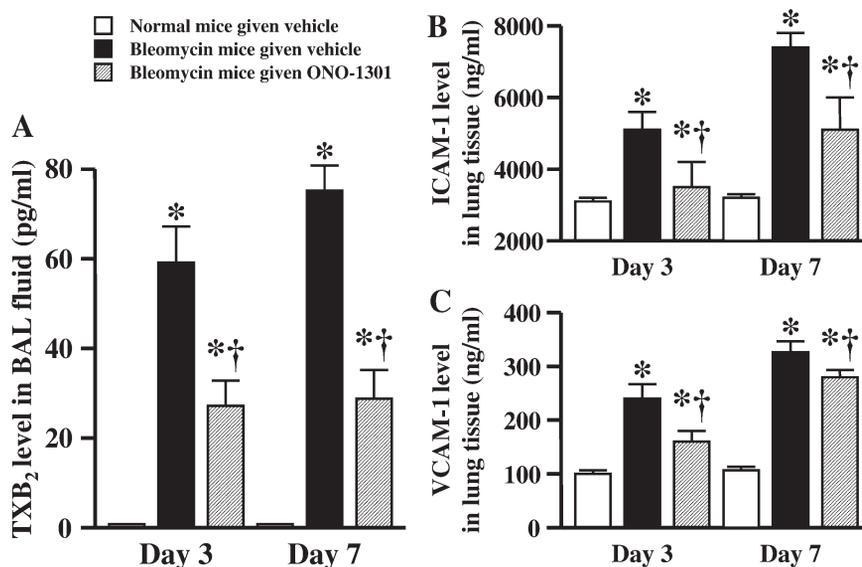
subcutaneous administration of ONO-1301 significantly decreased the hydroxyproline content in bleomycin-injected mice.

**Attenuation of lung inflammation.** The recovery rate of BAL fluid was  $>85\%$  in all groups. Total and differential cell counts were increased at 3 and 7 days after bleomycin injection (Fig. 3, A–D). However, subcutaneous administration of ONO-1301 significantly attenuated the increases in total cell count at 7 days and neutrophil count at 3 and 7 days after bleomycin injection. ONO-1301 administration tended to attenuate the increases in macrophage and lymphocyte counts, although these changes did not reach statistical significance. The total protein level was significantly increased at 3 and 7 days after bleomycin injection (Fig. 3E). However, the increase was significantly inhibited by ONO-1301.

**Inhibition of thromboxane synthesis.** The TXB<sub>2</sub> level in BAL fluid was significantly increased at 3 and 7 days after bleomycin injection (Fig. 4A), suggesting a pathological role of thromboxane in bleomycin-injected mice. Subcutaneous administration of ONO-1301 markedly inhibited the increase in TXB<sub>2</sub> level. The active TGF- $\beta$ 1 level in BAL fluid was significantly increased at 7 days after bleomycin injection (Sham group,  $148 \pm 6$ ; Vehicle group,  $251 \pm 8$ ; ONO-1301 group,  $238 \pm 9$  pg/ml). ONO-1301 did not significantly alter the active TGF- $\beta$ 1 level in BAL fluid.

**Inhibitory effect of ONO-1301 on ICAM-1 and VCAM-1 expression.** The lung ICAM-1 and VCAM-1 levels were significantly increased at 3 and 7 days after bleomycin injection (Fig. 4, B and C). ONO-1301 administration sig-

Fig. 4. A: effect of ONO-1301 administration on thromboxane B<sub>2</sub> (TXB<sub>2</sub>) level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 markedly inhibited the increase in TXB<sub>2</sub> level in BAL fluid. Data are means  $\pm$  SE. Effects of ONO-1301 administration on ICAM-1 (*B*) and VCAM-1 (*C*) levels in lung tissue at 3 and 7 days after bleomycin injection. \* $P < 0.05$  vs. Sham group, † $P < 0.05$  vs. Vehicle group.



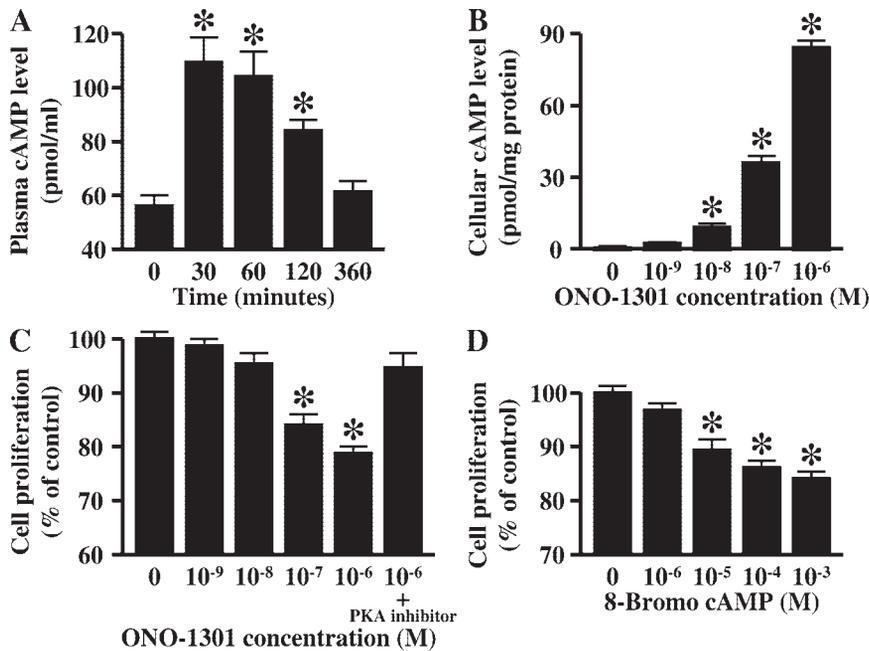


Fig. 5. A: time course of plasma cAMP level after a single subcutaneous administration of ONO-1301. B: dose-dependent effects of ONO-1301 on intracellular cAMP level. C: effect of ONO-1301 on fibroblast proliferation. ONO-1301 significantly reduced cell proliferation at concentrations of 10<sup>-7</sup> M or greater, and this inhibitory effect was attenuated by a protein kinase A (PKA) inhibitor. D: effect of 8-bromo cAMP on fibroblast proliferation. Data are means  $\pm$  SE. \**P* < 0.05 vs. Control.

nificantly attenuated the increases in ICAM-1 and VCAM-1 levels.

**Activation of the cAMP/PKA pathway.** A single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level (Fig. 5A). The increase lasted longer than 2 h. In vitro, ONO-1301 dose-dependently increased intracellular cAMP level in mouse lung fibroblasts (Fig. 5B). ONO-1301 significantly reduced proliferation of mouse lung fibroblasts at concentrations of 10<sup>-7</sup> M or greater, and this inhibitory effect was attenuated by a PKA inhibitor (Fig. 5C). The reduction in cell proliferation by ONO-1301 was reproduced by 8-bromo cAMP (Fig. 5D). Beraprost sodium (10<sup>-7</sup> M) significantly reduced fibroblast proliferation (86% of control). However, imidazole at different concentrations (10<sup>-6</sup>-10<sup>-9</sup> M) did not significantly regulate cell proliferation.

**Survival analysis.** Kaplan-Meier survival curves demonstrated that bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle (75 vs. 33% 21-day survival, log-rank test, *P* < 0.05, Fig. 6).

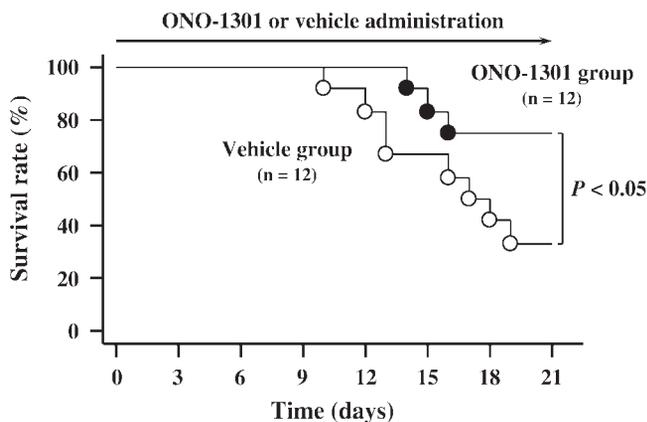


Fig. 6. Kaplan-Meier survival curves. Bleomycin mice treated with ONO-1301 (●) had a significantly higher survival rate than those given vehicle (○) (log-rank test, *P* < 0.05).

**Delayed therapy.** There were no significant differences in Ashcroft score and lung hydroxyproline content between the Vehicle group and ONO-1301 group at 28 days after bleomycin injection (data not shown).

## DISCUSSION

In the present study, we demonstrated that 1) repeated subcutaneous administration of ONO-1301 attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by decreases in Ashcroft score and lung hydroxyproline content, 2) ONO-1301 attenuated the increases in total cell count, neutrophil count, and total protein level in BAL fluid, and 3) ONO-1301 increased the survival rate in bleomycin-injected mice. We also demonstrated that 4) ONO-1301 decreased the TXB<sub>2</sub> level in BAL fluid and inhibited ICAM-1 and VCAM-1 expression in the bleomycin-treated lung, and 5) ONO-1301 inhibited lung fibroblast proliferation through activation of the cAMP/PKA pathway.

The balance between prostacyclin and TXA<sub>2</sub> plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. Recently, we have developed ONO-1301, a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase. Thus this compound was administered by subcutaneous injection twice a day.

Bleomycin induces lung inflammation followed by fibrosis when intratracheally injected in experimental animals (27). In the present study, subcutaneous administration of ONO-1301 significantly attenuated bleomycin-induced increases in total cell counts, neutrophil counts, and total protein level in BAL fluid. Earlier studies have shown that TXA<sub>2</sub> acts as a proinflammatory mediator via enhancement of pulmonary vascular permeability (18, 24) and neutrophil adhesion (33). In the present study, TXB<sub>2</sub>, a stable metabolite of TXA<sub>2</sub>, was significantly increased after bleomycin injection, which is consistent with previous studies (2, 8). However, ONO-1301 markedly

inhibited the increase in TXB<sub>2</sub> level in BAL fluid. Thus one of the possible mechanisms by which ONO-1301 attenuates lung inflammation may be mediated by inhibition of TXA<sub>2</sub> synthesis. A recent study has shown that a TXA<sub>2</sub> receptor agonist enhances the expression of adhesion molecules by human vascular endothelial cells (12). In the present study, ONO-1301 significantly inhibited ICAM-1 and VCAM-1 expression in the bleomycin-treated lung. Adhesion molecules including ICAM-1 and VCAM-1 have been shown to contribute to bleomycin-induced pulmonary fibrosis by mediating the accumulation of leukocytes (9, 16, 19, 23). These findings suggest that ONO-1301 may attenuate lung inflammation at least in part through inhibition of ICAM-1 and VCAM-1 expression.

Lung fibroblasts play an important role in the development of fibrosis in the lung (22, 25, 31). Prostaglandins are known to have various functions on lung fibroblasts via an elevation of intracellular cAMP level (3, 14–17). In the present study, a single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level in mice. In vitro studies demonstrated that ONO-1301 dose-dependently increased intracellular cAMP level in mouse lung fibroblasts and that this compound dose-dependently inhibited fibroblast proliferation. The inhibitory effect of ONO-1301 was reproduced by 8-bromo cAMP, a cAMP analog, and attenuated by a PKA inhibitor. These results suggest that ONO-1301 directly inhibits fibroblast proliferation at least in part through activation of the cAMP/PKA pathway. Dussaubat et al. (5) have demonstrated that imidazole, a thromboxane synthesis inhibitor, decreases bleomycin-induced acute lung inflammation, but it does not affect pulmonary fibrosis at later points. In the present study, a prostacyclin analog, but not a thromboxane synthesis inhibitor, significantly reduced fibroblast proliferation. These results suggest that the inhibitory effect of ONO-1301 on fibroblast proliferation may be mediated mainly by its prostacyclin-like activity. TGF- $\beta$ , especially TGF- $\beta$ 1 plays an important role in the pathogenesis of pulmonary fibrosis (7, 26, 32). In the present study, ONO-1301 did not significantly alter the active TGF- $\beta$ 1 level in BAL fluid. Previous studies have shown that a prostacyclin agonist suppresses TGF- $\beta$ -induced connective tissue growth factor, a potent profibrotic mediator, expression in part through activation of the cAMP/PKA pathway (28, 29). Thus it is interesting to speculate that ONO-1301 attenuated the development of pulmonary fibrosis through suppression of connective tissue growth factor.

In the present study, ONO-1301 significantly improved survival in bleomycin-injected mice. ONO-1301 inhibited lung inflammation and lung fibroblast proliferation. As a result, ONO-1301 may have beneficial effects on survival in bleomycin-injected mice. Unfortunately, we could not observe significant differences in fibrotic changes between the Vehicle group and ONO-1301 group when we administered ONO-1301 after fibrosis was established. These results imply that ONO-1301 may be insufficient to reverse established pulmonary fibrosis.

In conclusion, subcutaneous administration of ONO-1301, a novel long-lasting prostacyclin agonist, attenuated the development of bleomycin-induced pulmonary fibrosis and improved survival in mice. The beneficial effects were mediated at least in part by inhibition of TXA<sub>2</sub> synthesis and activation of the cAMP/PKA pathway. Thus administration of this com-

pound may be a new therapeutic strategy for the treatment of pulmonary fibrosis.

#### ACKNOWLEDGMENTS

The authors thank Yuki Isono, Natue Sakata, and Manami Sone for excellent technical assistance.

#### GRANTS

This work was supported by grants from Ono Pharmaceutical Co., Ltd.

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