Effects of Subtype-selective E Prostanoid Receptor Agonists on Bleomycin-induced Pulmonary Fibrosis in Rats

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Background : Idiopathic pulmonary fibrosis (IPF) is a fatal lung disease with limited treatment options and a poor prognosis. *In vitro* research has shown that prostaglandin (PG) E_2 can suppress pulmonary fibrosis via cAMP production. EP2 and EP4, which are subtypes of the receptors for PGE_2, are involved in cAMP production. The present study was designed to examine the effects of EP2 and EP4 agonists on bleomycin (BLM)-induced pulmonary fibrosis in rats.

Materials and Methods : The EP2 and EP4 agonists were subcutaneously administered to BLM-induced pulmonary fibrosis rats for 21 days. The lung weight, mRNA expressions of transforming growth factor (TGF)-β1 and procollagen genes, and degree of pulmonary fibrosis were compared between EP2 agonist, EP4 agonist, vehicle, and pirfenidone and nintedanib administered groups. We also examined the EP2 and EP4 expressions in human lung tissues with IPF and in rat lung tissues with BLM-induced pulmonary fibrosis by immunohistochemical staining. The human lung tissues with IPF were obtained from autopsy cases.

Results : The EP2 agonist significantly suppressed the lung weight gain and inhibited the mRNA expressions of TGF-β1 and procollagen genes. In addition, the fibrosis scores and hydroxyproline content tended to be lower in the EP2 agonist administered group. However, the EP4 agonist did not show such evidence in suppression of fibrosis. The enhanced expressions of EP2 and EP4 were demonstrated in both human and rat lung tissues with fibrosis relative to those in normal lung.

Conclusions : The EP2 agonist may become a novel therapeutic agent for IPF. *Shinshu Med J 67 : 183—195, 2019*

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Key words : pulmonary fibrosis, E prostanoid receptor agonists, cyclic adenosine monophosphate, transforming growth factor-β1, bleomycin-induced pulmonary fibrosis

I Introduction

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive, fibrosing, interstitial pneumonia of unknown causes and the prognosis is very poor. Currently, pirfenidone and nintedanib are clinically used in Japan for its treatment. Although these drugs significantly improved the standardized mean difference of change in forced vital capacity[1)2)], recent meta-analysis did not show significant improvement in mortality[3)]. Development of new anti-fibrotic drugs is expected to improve the prognosis of patients with IPF. Prostaglandin (PG) E_2, the major prostanoid in the lung, is an important antifibrotic lipid mediator. PGE_2 levels were reduced in the lungs of patients with IPF[4)]. Preventive effects of PGE_2 on pulmonary fibrosis have been studied in an animal model, bleomycin (BLM)-induced pulmonary fibrosis[5)6)]. Administration of PGE_2 to BLM-induced pul-

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monary fibrosis in mice suppressed collagen deposition and reduced mortality[5)6)]. It was demonstrated that administration of PGE_2 increased cAMP level with preventive effects of myofibroblast differentiation and inhibition of extracellular membrane production of pulmonary fibroblasts[7)]. Four E prostanoid receptor (EP) subtypes (EP1, EP2, EP3 and EP4) have been identified as receptors for PGE_2. EP2 and EP4 are coupled to Gs protein and can increase the intracellular cAMP level[8)]. We have developed an EP2 agonist[9)] and an EP4 agonist[10)] as candidates for novel antifibrotic agents. In the present study, we examined the pharmacological effects of the EP2 agonist and the EP4 agonist on BLM-induced pulmonary fibrosis in rats. We speculate that the expressions of EP2 and EP4 in lung tissues of rats with pulmonary fibrosis are fundamentaly required in the therapeutic target of the EP2 agonist and EP4 agonist on IPF. The immunohistological staining was performed in lung tissues of human IPF patients as well as of BLM-induced pulmonary fibrosis in rats in order to confirm the expressions of EP2 and EP4 in lung tissues. The present study was designed to evaluate the pharmacological potential of the EP2 and EP4 agonists on human IPF.

Ⅱ Materials and Methods

A Efficacy evaluation of EP2 and EP4 agonists in BLM-induced pulmonary fibrosis rats

1 Animals and reagents

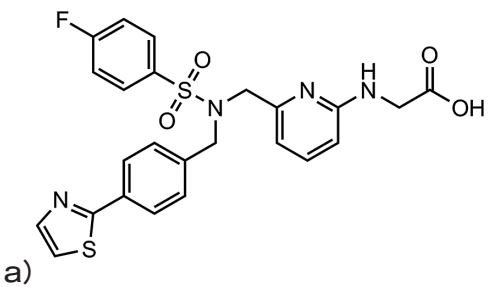
All experimental procedures were conducted according to Shinshu University Animal Experimental Rules, reviewed according to the guidelines of the National Regulation and Animal Care Committee, and approved by the Shinshu University Animal Research Committee (Authorization number : 270038, approval date : July 1st, 2016). The 48 Wister-Hann 9-week rats were purchased from Japan CRLs. We purchased BLM from Nippon Kayaku (Tokyo, Japan), midazolam from Fuji Pharma Co. (Tokyo, Japan), medetomidine from Kyoritsuseiyaku Co. (Tokyo, Japan) and butorphanol from Meiji Seika Pharma Co. (Tokyo, Japan). We purchased pirfenidone from Tokyo Chemical Industry Co. (Tokyo, Japan) and nintedanib from eNOvation Chemicals LLC (NJ, USA). The EP2 agonist and EP4 agonist were synthesized by Kissei Pharmaceutical Co. Ltd (Hotaka, Japan). The structural formula of the EP2 agonist is shown in **Fig. 1a** and that of the EP4 agonist in **Fig. 1b**. The EC_{50} of each compound was calculated as follows. HEK293T cells forcibly expressing rat EP2 and EP4 were stimulated with EP2 agonist, EP4 agonist and PGE_2 and analyzed according to the protocol of cAMP screening system (Thermo Fisher Scientific K. K.), using GraphPad Prism (GraphPad Software, California, USA) analyzed concentration effect data. Pharmacokinetics, plasma protein binding of the EP2 agonist and EP4 agonist in SD rats were obtained in studies conducted by Kissei Pharmaceutical Co. Ltd and are shown in **Fig. 1c, d**.

2 Study protocol

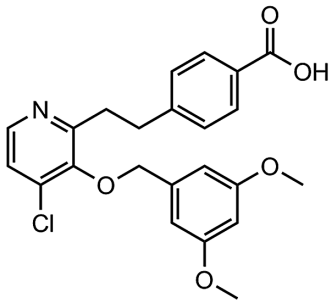
The experimental design is shown in **Fig. 2**. The 10-week rats were divided into 6 groups of 8 rats each. After intraperitoneal administration of mixed anesthesia (1 mg/kg midazolam, 0.2 mg/kg medetomidine, and 2.5 mg/kg butorphanol), the rats were intratracheally administered with 2.2 mg/kg BLM dissolved in 100 μL PBS. The sham control rats received intratracheally PBS only. On the next day, the administration of drugs, 0.3 mg/kg EP2 agonist (BLM + EP2), 0.3 mg/kg EP4 agonist (BLM + EP4), 30 mg/kg pirfenidone (BLM + pirfenidone), and 1 mL/kg vehicle (50 % PEG400, 50 % DMSO) (BLM + vehicle), were performed by subcutaneous injection three times a day, and 50 mg/kg nintedanib (BLM + nintedanib) was administered orally once a day.

The dosage of EP2 agonist and EP4 agonist were set at 0.3 mg/kg subcutaneous injection 3 times per day based on the pharmacokinetics and EC_{50} data.

The AUC for pirfenidone 600 mg oral single dose (single dose in clinical practice) to adult males, described in the interview form[11)], was 37.03 μg・hr/mL and Cmax was 10.57 μg/mL. The AUC determined by a single dose administration of pirfenidone 30 mg/kg for S/D rats conducted by Kissei Pharmaceutical Co. Ltd. was 33.79 μg・hr/mL and Cmax was 23.39. Because blood concentrations in clinically applied doses in human were maintained, the dosage and dose of pirfenidone in the present study was set to 30 mg/kg



a)



b)

c) Pharmacokinetic study of EP2 and EP4 agonists in rats

	EP2 agonist	EP4 agonist
C_{max} (ng/mL)	771.3	1511
T_{max} (min)	50.0	50.0
$AUC_{(0\text{-}X)}$ (ng・min/mL)	172,330.3	320,213
Protein binding (%) rat plasma 37℃, 10min	99.88	99.91

Cmax: maximum serum drug concentration,

Tmax: Time to reach maximum serum concentration,

AUC(0-X): Area under the concentration–time curve over x min

d) Functional assay of transient expression in HEK293T cell

	EP2 agonist	EP4 agonist
Rat EP2 EC_{50} (nM)	0.010	117.8
Rat EP4 EC_{50} (nM)	0.12	0.0005

Fig. 1

a Structural formula of EP2 agonist
b Structural formula of EP4 agonist
c Pharmacokinetic study of EP2 and EP4 agonists in rats
d Functional assay of transient expression in HEK293T cell

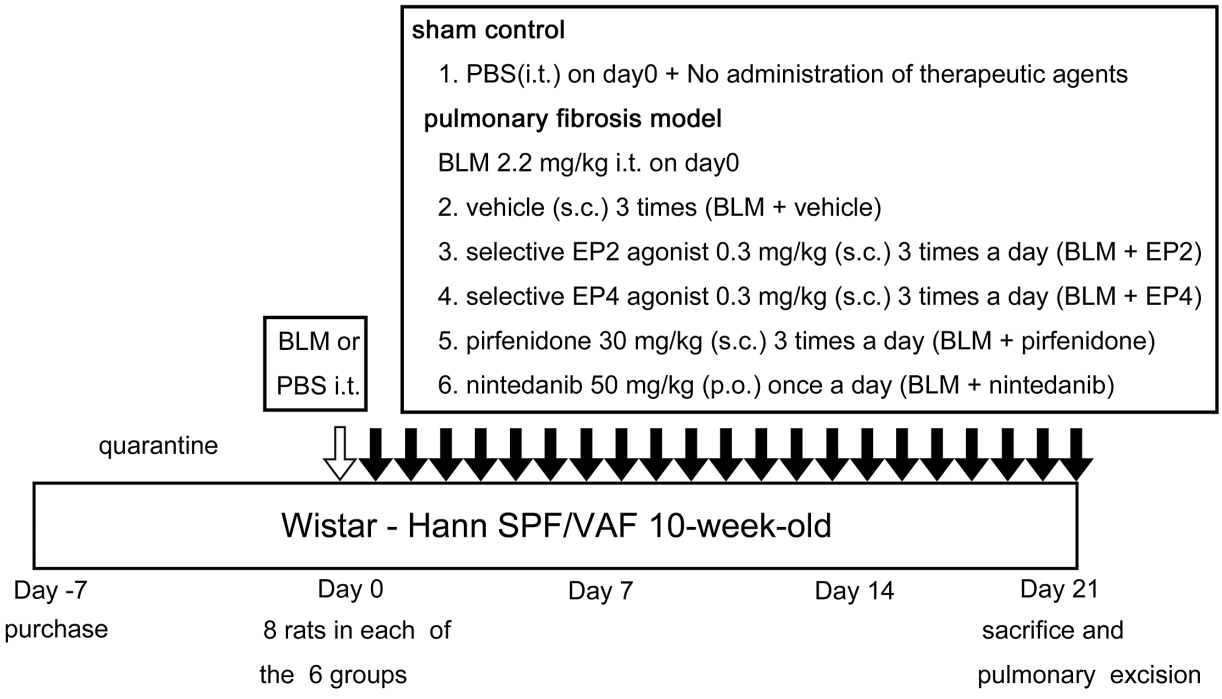


Fig. 2

Study design for examining the antifibrotic effects of the EP2 and EP4 agonists on bleomycin (BLM)-induced pulmonary fibrosis rats.

subcutaneous injection three times a day.

The dosage of nintedanib was set according to the method in the study for BLM-induced pulmonary fibrosis in rats performed by Wollin et al.[12]

Body weight measurements were taken daily before the first dose. We euthanized the rats by abdominal aortic dissection under deep anesthesia on day 21 of the administration of intervention agents. We removed both lungs to evaluate the efficacy of intervention by the agents.

3 Measurement of lung weight

The right lung was removed and the weight was measured. The lung specimens were quickly frozen with liquid nitrogen and stored at −30℃ until the measurements of hydroxyproline content and quantitative real-time polymerase chain reaction (qRT-PCR).

4 Histological analysis

The left lung was removed, fixed by 10 % neutral-buffered formaldehyde solution and embedded in paraffin. The sections were stained with Masson's trichrome and examined by light microscopy. The fibrosis score was evaluated using the modified Ashcroft's score[13]. The lung section was examined at a magnification of 100×, and each field was visually scored from 0 points (normal lung) to 8 points (complete obstruction with fibroma mass) under light microscopy. The average score was calculated with all the observed fields for an entire lung.

5 Measurement of hydroxyproline content

A sample of right lung tissue was homogenized with 1,000 mg of lung tissue in 1 mL of PBS. Hydroxyproline content was measured by the assay kit (Chondrex Inc, WA, USA) following measurement protocol.

6 mRNA expressions of the TGF-β1, procollagen1a1 (Col1a1), and Col1a2 genes

The total RNA was extracted from right lung tissues using a RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's designated regimen. We synthesized cDNA from a 100ng total RNA template via reverse transcription by using a Prime Script RT Enzyme Mix I (TakaraBio Inc., Japan).

The mRNA levels were quantified using the qRT-PCR system (Applied Biosystems StepOnePlus; Thermo Fisher Scientific K.K.). The cDNAs of the rat Col1a1, Col1a2, and TGF-β1 were amplified from single-stranded cDNA by PCR using Taq DNA Polymerase with 0.4 μM of each primer. The PCR primers of the rat TGF-β1, Col1a1, Col1a2 genes, and 18S rRNA are described in **Table 1**. The qRT-PCR was performed using 10 μmol samples consisting of 200 nM of each primer, 12.5 μL of SYBR premix Ex Taq (TakaraBio), and 10 ng of template cDNA.

The experimental PCR protocol required 30 s of an initial denaturation at 96℃ followed by 40 cycles of 5 s of denaturation at 96℃, 30 s of annealing at 60℃, and 60 s of extension at 60℃.

B Confirmation of EP2 and EP4 expressions in the lung tissues

1 Rat lung tissues

The lung tissues of rats with administration of PBS intratracheally were used as negative control. The lung tissues of rats in BLM＋vehicle group were used

Table 1 The primer sequence of cDNA used in the present study

cDNA, (ID)	primers	sequence
TGF-β1	forward	5'-CATTGCTGTCCCGTGCAGA-3'
(RA059245)	reverse	5'-AGGTAACGCCAGGAATTGTTGCTA-3'
Col1a1	forward	5'-CTGCATCAGGGTTTCAGAGCAC-3'
(RA069752)	reverse	5'-TCCACATGCTTTATTCCAGCAATC-3'
Col1a2	forward	5'-CTGGATTGACCCTAACCAAGGATG-3'
(RA069679)	reverse	5'-TTGACAGGTTGGGCCGGA-3'
18S rRNA	forward	5'-AAGTTTCAGCACATCCTGCGAGTA-3'
(RA015374)	reverse	5'-TTGGTGAGGTCAATGTCTGCTTTC-3'

for the indication of pulmonary fibrosis.

2 Human lung tissues

Approval regarding using human lung materials in this study was obtained from the Shinshu University Ethics committee (Authorization number : 2730, approval date : May 12th, 2014).

The lung tissues with pulmonary fibrosis were obtained from autopsy cases of IPF in 2008 and 2009. The normal lung tissues for the control were obtained from resected lung lobes after lung lobectomy performed for the treatment of lung cancer in the Department of Thoracic Surgery of Shinshu University. Tissues were fixed in formalin and embedded in paraffin.

3 Immunohistochemical staining

We used Histofine SAB-PO kit (Nichirei Biosciences Inc., Tokyo, Japan) for immunostaining and performed the procedure following the protocol. The SAB-PO kit contains biotin-labeled anti-mouse IgG + IgA + IgM rabbit antibody as a second antibody and 3, 3-diaminobenzidine (DAB) as a chromogenic substrate. The anti-EP2 rabbit monoclonal antibody (ab 167171, Abcam, Cambridge, UK) was diluted at 1 : 1,250 with PBS and the anti-EP4 rabbit polyclonal antibody (ab 133170, Abcam) was diluted at 1 : 50 with PBS and used as the primary antibody.

C Statistical analysis

Data were expressed as the mean ± standard deviation (SD) and processed by using GraphPad Prism 7 (GraphPad Software, Inc. USA). The significance of differences between each group was evaluated by one-way ANOVA with post-hoc tests. A value of $P < 0.05$ was considered to indicate statistical significance.

Ⅲ Results

A Efficacy evaluation of the EP2 and EP4 agonists in the BLM-induced pulmonary fibrosis rats

1 Histopathological effects of the intervention compounds

The alveolitis and patchy fibrosis with destruction around the bronchus (representing the part surrounded by the red ellipse) were observed in the rat BLM-induced lung fibrosis model. In the sham control, only normal lung findings were observed **(Fig. 3a)**. The pathological findings were improved in BLM + EP2 **(Fig. 3c)** and BLM + nintedanib **(Fig. 3f)** relative to those in BLM + vehicle **(Fig. 3b)**. On the other hand, there was no pathological improvement in BLM + EP4 **(Fig. 3d)** and BLM + pirfenidone **(Fig. 3e)**.

The mean fibrosis scores in groups of the sham control, BLM + vehicle, BLM + EP2, BLM + EP4, BLM + pirfenidone, and BLM + nintedanib were 0.07 ± 0.06, 3.10 ± 0.75, 2.88 ± 0.74, 3.41 ± 0.60, 3.62 ± 0.81, and 2.64 ± 0.59, respectively. The mean fibrosis scores of the BLM + EP2 and BLM + nintedanib were lower than that of the BLM + vehicle ; however, no statistical significance was observed. **(Fig. 4a)**.

2 Effects on lung weights

The average lung weight of each experimental group is shown in **Fig. 4b**. The lung weight of BLM + vehicle was significantly higher than that of the sham control. The average lung weights of BLM + EP2 and BLM + nintedanib were significantly lower than that of BLM + vehicle. However, the EP4 and pirfenidone did not show effects on the lung weights of rats with BLM-induced pulmonary fibrosis.

3 Hydroxyproline content in lung tissues

The hydroxyproline content in lung tissue is shown in **Fig. 4c**. The content was 22.7 ± 13.2 μg/mL in the sham control, whereas it was increased to 85.3 ± 52.8 μg/mL in the BLM + vehicle. The hydroxyproline content was slightly suppressed to 71.0 ± 43.6 μg/mL in the BLM + EP2 ; however, there was no significant difference between the BLM + EP2 and BLM + vehicle. No suppressive effect was observed in the other groups, in which the hydroxyproline contents were 93.2 ± 61.4 μg/mL in the BLM + EP4, 96.0 ± 52.1 μg/mL in the BLM + pirfenidone, and 85.0 ± 52.0 μg/mL in the BLM + nintedanib.

4 The mRNA expressions of TGF-β1, Col1a1, and Col1a2 genes

The TGF-β1 mRNA was significantly increased in BLM + vehicle compared to that in the sham control. On the other hand, it was significantly suppressed in BLM + EP2, BLM + EP4, BLM + pirfenidone and BLM + nintedanib compared with that in BLM + vehicle **(Fig. 5a)**.

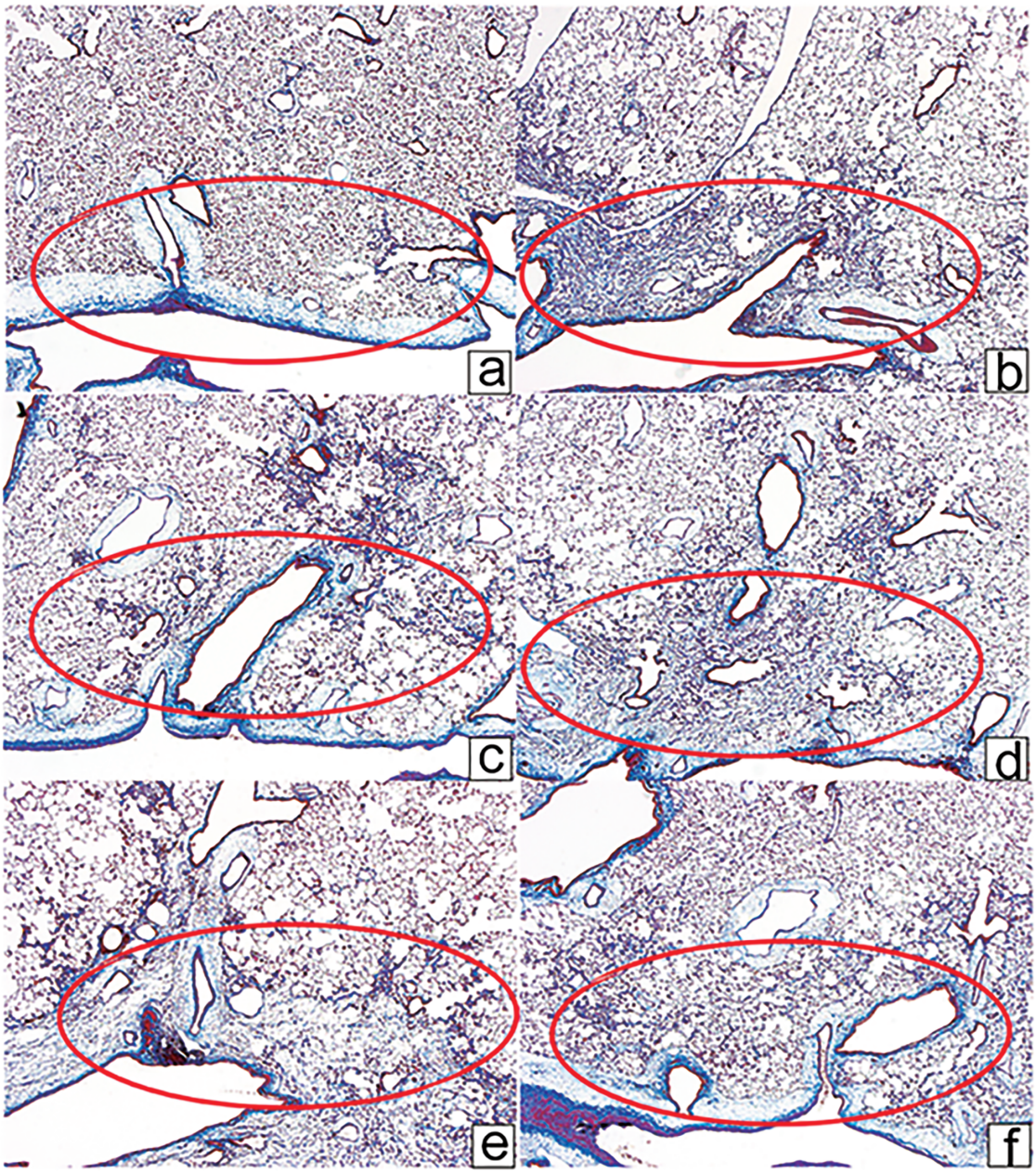


Fig. 3
Histological observation of the left lung (Masson trichrome ; original magnification ×20).
a PBS only, as a sham control b BLM + vehicle c BLM + EP2 agonist
d BLM + EP4 agonist e BLM + pirfenidone f BLM + nintedanib.

The expressions of Col1a1 mRNA were significantly enhanced in BLM + vehicle compared with those in the sham controls. The mRNA expression of Col1a1 gene was significantly suppressed in BLM + EP2 and BLM + pirfenidone compared with that in BLM + vehicle **(Fig. 5b)**. The expression of Col1a2 mRNA was increased in BLM + vehicle compared to those in the sham controls without significant difference. The mRNA expression of Col1a2 gene was significantly suppressed in BLM + EP2, BLM + pirfenidone, and BLM + nintedanib compared with that in BLM + vehicle **(Fig. 5c)**.

B Confirmation of EP2 and EP4 expressions in the lung tissues

1 Immunostaining of EP2 and EP4 in lung tissues of experimental rats

The immunostaining showed very strong expressions of the EP2 **(Fig. 6a)** and EP4 **(Fig. 6c)** in the

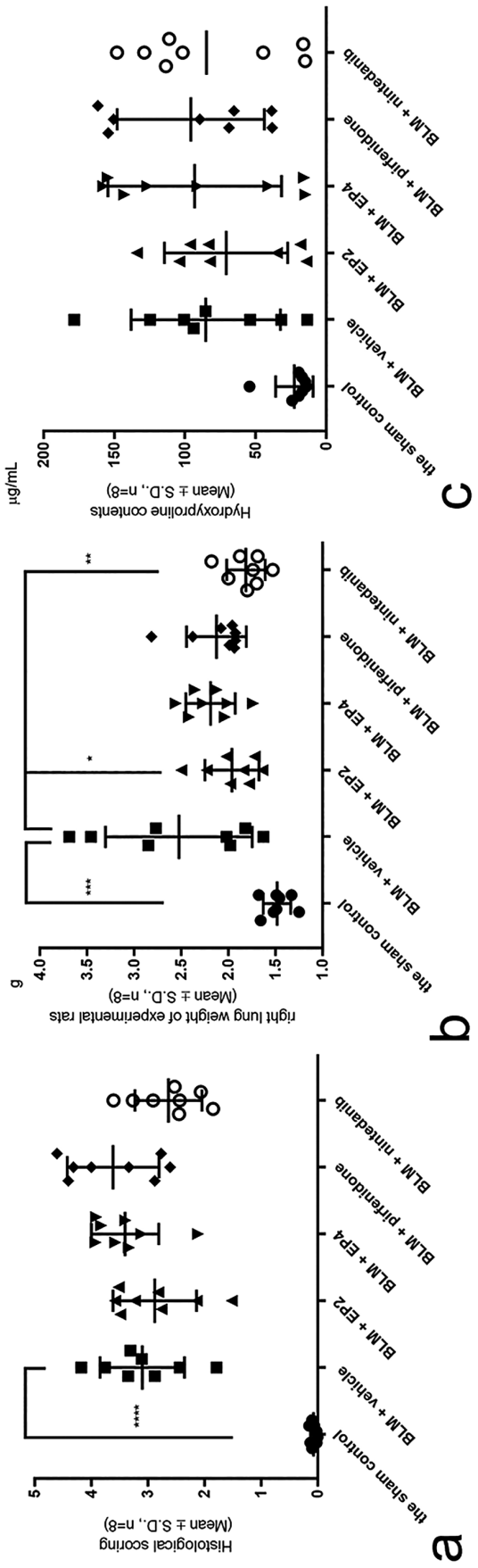


Fig. 4

a Histological score by modified Aschoff's score method : **** indicates $p<0.0001$ vs. BLM + vehicle group

b The right lung weight of experimental rats : * indicates $p<0.05$ vs. BLM + vehicle, ** indicates $p<0.005$ vs. BLM + vehicle and *** indicates $p<0.001$ vs. BLM + vehicle.

c Hydroxyproline contents in 1 gram of right lung tissue

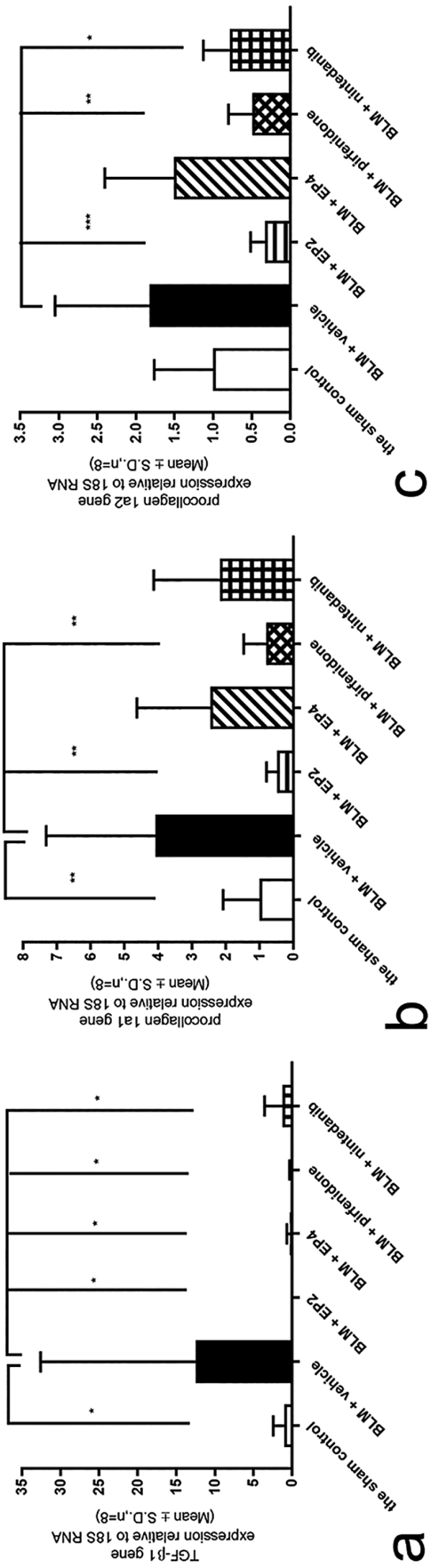


Fig. 5

a The mRNA expression of TGF-β1

b The mRNA expression of procollagen 1a1

c The mRNA expression of procollagen 1a2 : *indicates $p<0.05$ vs. BLM + vehicle, **indicates $p<0.005$ vs. BLM + vehicle and ***indicates $p<0.001$ vs. BLM + vehicle

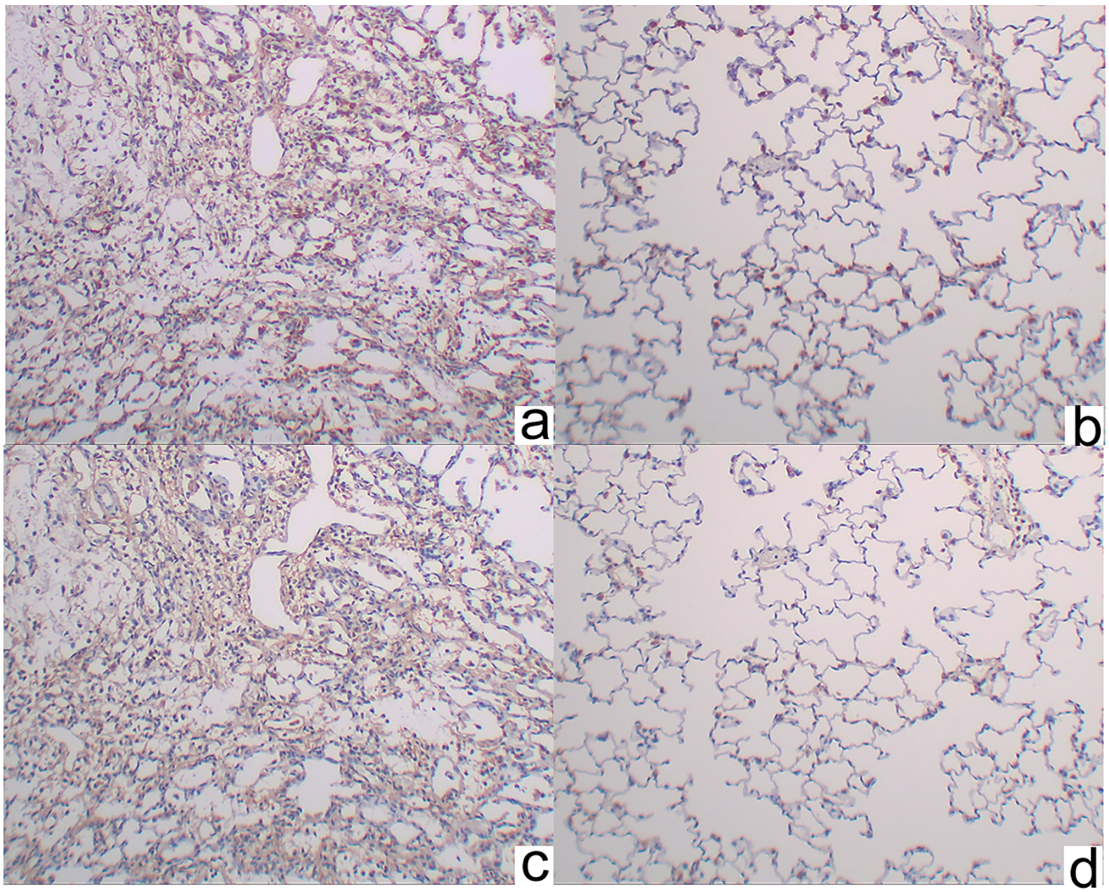


Fig. 6
Immunostaining of rat lung ; original magnification ×100.
a The EP2 of BLM-induced pulmonary fibrosis rat
b The EP2 of rat administered with PBS i.t.
c The EP4 of BLM-induced pulmonary fibrosis rat
d The EP4 of rat administered with PBS i.t. The parts with EP2 expression reacted with DAB and showed a brown color. The parts without expression of EP2 and EP4 exhibited a blue color.

lung tissues of rats with BLM-induced pulmonary fibrosis compared to those in the negative control **(Fig. 6b, d)**.

2 Immunostaining of EP2 and EP4 in human lung of IPF

The immunostaining showed that the EP2 was positive in human IPF tissues **(Fig. 7a)** but negative in normal lung tissues **(Fig. 7b)**. On the other hand, the EP4 showed weak expression in the alveolar epithelium of IPF tissues **(Fig. 7c)**, but negative expression in the normal lung tissues **(Fig. 7d)**.

Ⅳ Discussion

Among the four subtypes of the PGE_2 receptor, EP2 and EP4 are coupled to G protein to increase cAMP. Increased intracellular cAMP levels were reported to suppress the inductive activity of TGF-β to attenuate fibroblast migration[14]. TGF-β1 is a key cytokine whose sustained production causes the development of tissue fibrosis. Repeated injury with continued autoinduction of TGF-β1 overrides the normal termination signals, thus creating a chronic vicious circle of TGF-β1 overproduction[15]. In the present study, the mRNA production of TGF-β1 gene was suppressed by the administration of the EP2 and EP4 agonists, suggesting a hindrance of the vicious cycle of TGF-β1 overproduction. The suppressions of EP2 on the expressions of mRNA of TGF-β1 gene and procollagen gene in the BLM-induced pulmonary fibrosis rats were equivalent to

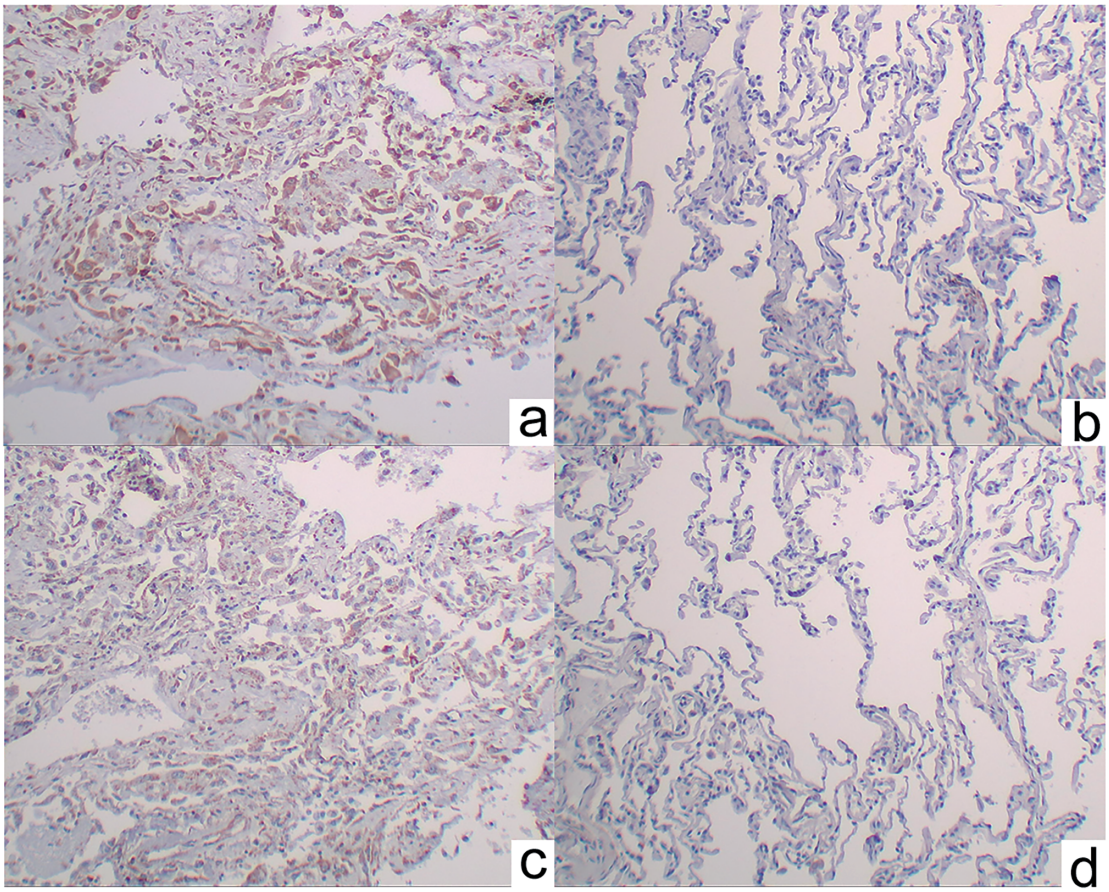


Fig. 7
Immunostaining of human lung ; original magnification ×100.
a The EP2 was expressed in human lung of IPF with a brown color.
b The EP2 was not expressed in human normal lung tissue with a blue color.
c The EP4 was expressed in human lung of IPF, d) The EP4 was not expressed in normal lung tissue.

the suppression of nintedanib in the rats with pulmonary fibrosis. Our study reproduced the same results for BLM＋nintedanib as those reported by Wollin[12]. Despite the significant reductions in mRNA of TGF-β1 and procollagen genes, there was no significant difference in the mean fibrosis scores and hydroxyproline contents between the BLM＋EP2 and BLM＋vehicle groups.

A plausible explanation is the problem of the evaluation method. In the present study, the pulmonary fibrosis in rats was induced by intratracheal administration of BLM. Thus, the fibrosis was remarkably developed around the bronchus. The BLM ＋vehicle formed strong fibrosis around the bronchiole, which was obviously improved with the administration of EP2 and nintedanib. The fibrosis score was calculated by the average score of about 100 fields under light microscopic observation for each rat. The fact is that the pathological parts of the fibrosis were not evenly distributed on the whole lung fields, with focus on some of the lung fields and scattering on the other fields. Thus, the significant differences in the fibrosis score vanished with the average calculation due to the large SD in the statistics. The reason for no significant difference of hydroxyproline contents between the BLM＋EP2 and BLM ＋vehicle groups was similar. In addition, the weight of the entire right lung was significantly lower in BLM＋EP2 and BLM＋nintedanib than in BLM＋vehicle. The weight of lung with fibrosis is heavier than that of normal lung. We believe that antifibrosis was achieved in the BLM＋EP2 and BLM＋nintedanib

groups.

In addition, significant differences in the Col1a1 mRNA expression and different tendencies of fibrosis were observed between the rats treated with EP2 and EP4 agonists. EP4 was found to have induced desensitization and internalization by stimulation of PGE_2[16]. Therefore, by stimulation with the same amount of PGE_2, the expression of cAMP in EP2-expressing cells was observed to be 7-fold as compared with EP4-expressing cells[17]. The production of cAMP inhibits collagen synthesis and prevents differentiation of fibroblasts into probiogenic myofibroblasts[18]. Therefore, the inhibitory effects of EP2/4 agonists on the procollagen mRNA can be explained. We speculate that a possible role of the cAMP level may be one of the causes of the difference in the efficacy between the EP2 and EP4 agonists in the present study. Moreover, it has been reported that another signaling pathway, which is independent of cAMP, could be present in EP4[16)19)20)].

Pirfenidone is clinically applied to treat patients with IPF. In the present study, pirfenidone failed to reduce the BLM-induced pulmonary fibrosis. Schaefer et al. reviewed experiments on the effects of pirfenidone on the animal model of BLM-induced pulmonary fibrosis[21] and stated in summary that the anti-fibrotic effect was mainly found in mice and hamsters[22)-25)]. In addition, pirfenidone was administered by oral administration in these studies. Recently, it was reported that oral administration of 50 mg/kg pirfenidone could reduce BLM-induced pulmonary fibrosis in rats[26]. In the present study, pirfenidone was administered to rats by 30 mg/kg subcutaneous injection three times a day. We believed that the dose would be approximately equal to that when orally administered to humans in clinical practice. However, our dose and animal setting or administration route may be related to the lack of preventive effects on BLM-induced pulmonary fibrosis in the present study. We need further experiments at various doses and oral administration of pirfenidone in BLM-induced pulmonary fibrosis.

The present study demonstrated that EP2 and EP4 were expressed in human and rat lung tissues with fibrosis but not normal lung tissues, which are noteworthy new findings in pulmonary fibrosis. The expression of the receptors suggested that EP2 and EP4 are related to the development of pulmonary fibrosis. However, we found that EP4 over-expression was not apparent compared with that of EP2. The lack of preventive effects of EP4 agonist on pulmonary fibrosis could be related the low expression in the lung. However, we could show that the EP2 agonist contributes to the prevention of fibrosis in an experimental model. We need further studies using experimental models and clinical samples ; however, the present study indicates that the EP2 agonist may be an effective therapeutic agent for IPF.

Conflicts of Interest

The authors have no conflicts of interest in the present study.

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