Treatment with both nintedanib and steroid in bleomycin-induced pulmonary fibrosis model mice exacerbated lung fibrosis

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Short Title: Nintedanib+steroid treatment exacerbates lung fibrosis

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Abstract

Background: In the clinical setting, the optimal timing of administration of nintedanib in the active or inactive phase of idiopathic pulmonary fibrosis (IPF) has not been clarified. Also, no consideration has been made of synergistic actions with steroids. **Objectives:** We used bleomycin-induced pulmonary fibrosis model mice to analyze the effects of the timing of nintedanib administration and synergistic actions in combination with steroid.

Method: In the bleomycin-induced pulmonary fibrosis mouse model, nintedanib was given before or after bleomycin administration and the effects on pulmonary fibrosis and fibroblast growth factors in bronchoalveolar lavage (BAL) fluid were analyzed. Then, treatment with both steroid and nintedanib was administered in the same model and the effects of combined use on lung fibrosis were analyzed. Severity of lung fibrosis was analyzed using the Ashcroft score. Fibroblast growth factors in BAL fluid were measured using Magnetic Luminex® assay and enzyme-linked immunosorbent assay.

Results: Pretreatment with nintedanib before administration of bleomycin, but not after administration of bleomycin, reduced the Ashcroft score (2.4±1.4 vs 4.9±1.3, $P<0.01$) and decreased TGF-β1 in BAL fluid (95.7±48.4 pg/mL vs 147.1±11.7 pg/mL, $P<0.05$).

Conversely, pretreatment with both steroid and nintedanib did not decrease the Ashcroft score compared with pretreatment of saline as a control (Score: 3.8±1.3 vs 4.6±1.1). There was also no synergistic effect of both steroid and nintedanib on pulmonary fibrosis.

Conclusions: Nintedanib may be useful when administered early in patients with IPF. Further, clinically, administration of combined nintedanib and steroid requires caution in view of the effects on pulmonary fibrosis and side effects.

Introduction

Idiopathic pulmonary fibrosis (IPF) is one of the idiopathic interstitial pneumonias (IIPs). It is a type of lung disease characterized by extensive fibrosis in the lungs and causes restrictive ventilatory impairment. Compared with other IIPs, IPF shows poor response to steroids and immunosuppressive drugs and carries a poor prognosis [1]. IPF is the most common of the IIPs.

The precise etiology is not yet known, but smoking is considered to be a risk factor [2]. In IPF, inflammation does not necessarily precede fibrosis, but with increased damage to the alveolar epithelium by various stimuli, excessive collagen deposition

required for repair ensues with an abnormal repair reaction, leading to the progression of fibrosis [3,4]. Interstitial thickening is considered to reduce oxygen uptake causing restrictive disorder (reduced vital capacity) due to decreased lung compliance; symptoms include dry cough and dyspnea on exertion [5, 6]. Transforming growth factor (TGF)-β is considered to play an important role in fibrosis, and it is known to cause epithelial-mesenchymal transition (EMT) to type II alveolar epithelium [7, 8]. It is also known to induce differentiation into fibroblasts and myofibroblasts [9]. There is no curative treatment for IPF and currently available treatments, except for lung transplantation, only slow progression [10].

Recently, pirfenidone was reported to suppress fibrosis, and it has been found to reduce deterioration in relation to forced vital capacity (FVC) and the 6-min walk test compared with placebo [11, 12]. Although the mechanism of action of pirfenidone is unclear, it is known to suppress TGF-β and tumor necrosis factor (TNF)-α in vitro [13]. More recently, nintedanib, a small molecule tyrosine kinase inhibitor (TKI) and indolinone derivative, has been used as a therapeutic agent for IPF [14, 15]. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived

growth factor (PDGF) promote angiogenesis and fibroblast proliferation [16~18]. Nintedanib acts on VEGF, FGF, and PDGF receptors [19] and thus inhibits PDGF, FGF, VEGF-stimulated proliferation and migration of lung fibroblasts and subsequently TGF-β-induced fibroblast transformation [19, 20]. However, in the clinical setting, the timing of administration of nintedanib in the active or inactive phase of IPF has not been clarified. Also, no consideration has been made regarding synergistic actions with steroids.

Here, we used a bleomycin-induced pulmonary fibrosis mouse model to analyze the effects of the timing of nintedanib administration and the synergistic action with steroid.

Materials and Methods

Animals

Nine-week old female C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). This study was approved by the Animal Ethics Committee of Dokkyo Medical University Saitama Medical Center (No. 767).

Effects of treatment with nintedanib before and after administration of bleomycin

To investigate the effect of nintedanib before and after inducing lung inflammation, we performed transesophageal treatment with nintedanib before or after intratracheal administration of bleomycin. Figure 1A shows the protocol. Saline (bleomycin[-]) (Group 1) or bleomycin sulfate (bleomycin[+]; 5 mg/kg body weight; Nippon Kayaku, Tokyo, Japan) (Group 2, 3, and 4) was given by intratracheal administration to mice anesthetized with medetomidine hydrochloride (Zenoaq, Fukushima, Japan), midazolam (Astellas Pharma Inc., Tokyo, Japan), and butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), as a mixture of the 3 anesthetics (10 mL/kg body weight) on day (D)1. Nintedanib (LC Laboratories, Boston, MA) (3 mg/kg body weight) with doses similar to humans was given by transesophageal administration before (D0) (Group 3) or after (D2) (Group 4) intratracheal administration of bleomycin. In contrast to treatment with nintedanib, transesophageal saline was administered after (D2) (Group 2) intratracheal administration of bleomycin. Subsequently, nintedanib (Group 1, Group 3,

and Group 4) or saline (Group 2) was given daily, twice a day from D2 to D6 and from D8 to D13. The dose of nintedanib was determined similar to the dose used in humans.

Effects of treatment with both nintedanib and steroid before and after administration of bleomycin

To investigate the effect of nintedanib with steroid before and after inducing lung inflammation, we performed transesophageal treatment with nintedanib and dexamethasone before or after intratracheal administration of bleomycin. Figure 2 shows the protocol. Groups 2, 3, and 4 were administered bleomycin to induce pulmonary fibrosis. Saline (bleomycin[-]) (Group 1) or bleomycin sulfate (bleomycin[+]; 5 mg/kg body weight) (Groups 2, 3, and 4) was given by intratracheal administration to mice using a mixture of the 3 anesthetics on D1. Both nintedanib (3 mg/kg body weight) and moderate dexamethasone (0.1 mg/kg body weight) when treated to humans was given by transesophageal administration before (D0) (Group 3) or after (D2) (Group 4) intratracheal administration of bleomycin. Alternatively, saline was given by transesophageal administration after (D2) (Group 2) intratracheal

119 administration of bleomycin. Subsequently, both nintedanib and dexamethasone
120 (Groups 1, 3, and 4) or saline (Group 2) was given daily, twice and once a day,
121 respectively, from D2 to D6 and from D8 to D13.
122
123 ***Bronchoalveolar lavage***
124 A single incision was made in the neck and the tissue covering the trachea was snipped
125 to expose the tracheal rings. Bronchoalveolar lavage (BAL) fluid was collected on D15
126 after bleomycin or saline administration; histological examination of the lungs was also
127 performed on D15. Using a 1.5 ml syringe, 0.5 ml was injected into the trachea once,
128 and BAL was performed 3 times. The BAL fluid sample was centrifuged at 400 g for
129 10 min at 4℃, and the supernatant was stored at -80℃ until analysis.
130
131 ***Concentration of FGF2, PDGF-AA, VEGF-A, and collagen in BAL fluid***
132 BAL fluid samples were concentrated by centrifugal ultrafiltration (Amicon Darmstadt,
133 Germany), which is used to concentrate low molecular weight components. The cut-off
134 value for molecular weight was 3000. BAL fluid levels of FGF2, PDGF-AA, VEGF-A

were measured by Magnetic Luminex Assay, Mouse Premixed Multi-Analyte Kit (R&D, Minneapolis, MN) using a multi-item simultaneous measurement device (Luminex Japan Corporation, Tokyo, Japan). The minimum detectable dose (MDD) was 41.3 pg/mL, 0.9 pg/mL, and 4.0 pg/mL, respectively. In addition, concentrations of TGF-β1 were measured using enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN) according to the manufacturer's instructions. In preliminary experiment, albumin concentration in BAL was measured for 5 mice in all groups presented this time, and corrected for the concentration of each protein (TGF-β, VEGF, PDGF-AA). However, since there was no significant difference, no correction by albumin concentration was performed in this study. The MDD was 4.6 pg/mL. Collagen measurements were determined using the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, UK) [21]. The collagen concentration was measured at 549 nm on a spectrophotometer.

Histopathological analysis

The right lung tissues were preserved in order to develop this study in the future, and the left lung was used in this study. To fix the lung, paraformaldehyde in phosphate

buffer was perfused into the left lung at a constant pressure. After fixation, lung tissues were embedded in paraffin and sectioned with a microtome at a thickness of 3-5 μm. Sections were mounted on slides and stained with hematoxylin and eosin (HE), Elastica-Masson (EM), and for immunohistochemistry (IHC). For each mouse, three sections of the whole lung stained with HE and EM were selected randomly. To analyze the severity of lung fibrosis, grading was scored on a scale from 0 to 8, using the average of microscope field scores, as described previously [22]. Biological micrographs were taken with a digital camera for microscope (model DP73; Olympus Corporation, Tokyo, Japan) with a ×10 and ×40 lens.

Immunostaining analysis

Paraffin-embedded sections of tissue were deparaffinized and dehydrated following antigen retrieval with citric acid buffer, as described previously [23]. Slides were first incubated with blocking IgG solution for 1 h and then overnight with rabbit anti-TGF-β1 antibody (1:100 dilution; Abcam), rabbit anti-FGF2 antibody (1:200 dilution; Bioss, Woburn, MA), anti-VEGF-A antibody (1:200 dilution; Abcam), or

rabbit anti-PDGF-AA antibody (1:400 dilution; Bioss). The secondary antibody used was a labeled polymer containing peroxidase and Fab′ anti-IgG bound to an amino acid polymer (Histofine mouse stain kit; Nichirei Bioscience, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical significance was determined with the Mann-Whitney *U* test for each group. *P* values < 0.05 were considered significant. JMP software (Version 11.0; SAS Institute, Cary, NY) was used for statistical analyses.

Results

Effects of nintedanib before and after administration of bleomycin: histopathological analysis and TGF-β1, FGF2, PDGF-AA, VEGF-A, and collagen concentrations in BAL fluid

We first performed histopathological analysis (Figure. 3A to Figure. 3H) and measured concentrations of collagen (Figure. 3J), TGF-β1 (Figure. 3K), PDGF-AA (Figure. 3L),

and VEGF-A (Figure. 3M) in BAL fluid on D15 of lung treatment with nintedanib before and after administration of bleomycin in. The Ashcroft score as an index of lung fibrosis (Figure. 3I) was significantly reduced in Group 3 compared with Group 2 (2.4±1.4 vs 4.9±1.3, $P<0.01$) but not in Group 4 (3.6±1.5, $P=0.106$) compared with Group 2.

The concentration of collagen in BAL fluid was significantly reduced in Group 3 compared with Group 2 (4.4±3.0 μg/mL vs 10.0±6.5 μg/mL, $P<0.05$; Figure. 3J) but not in Group 4 (7.0±3.0 pg/mL) compared with Group 2. In addition, the concentration of TGF-β1 in BAL fluid was significantly reduced in Group 3 compared with Group 2 (95.7±48.4 pg/mL vs 147.1±11.7 pg/mL, $P<0.05$; Figure. 3K) but not in Group 4 (120.2±26.4 pg/mL) compared with Group 2. The concentrations of PDGF-AA and VEGF-A in BAL fluid were not reduced in either Group 4 or Group 3 compared with Group 2. FGF2 was not detected in BAL fluid in any of the groups (data not shown).

Bleomycin-induced pulmonary fibrosis: IHC analysis

198 We performed IHC analysis on D15 after administration of bleomycin (Figure 4).
199 TGF-β1 were expressed in lung fibroblasts (Figure 4B). Although FGF2 was not
200 detected in BAL fluid, it was expressed in lung fibroblasts (Figure 4D). PDGF-AA
201 (Figure 4F) and VEGF-A (Figure 4H) were expressed mainly in endothelial and
202 epithelial cells.
203
204 ***Effects of both steroid and nintedanib before and after administration of bleomycin:***
205 ***Histopathological analysis, and collagen concentrations in BAL fluid***
206
207 Finally, we performed histopathological analysis on D15 of treatment with both steroid
208 and nintedanib before and after administration of bleomycin (Figure. 5A to Figure.5H).
209 Ashcroft score (Figure. 5I) was not reduced in Group 3 (3.8±1.3) or Group 4 (4.1±1.3)
210 compared with Group 2 (4.6±1.1). In addition, concentration of collagen (Figure. 5J) in
211 BAL fluid was not reduced in Group 3 (11.1±4.2) or Group 4 (10.2±4.0) compared with
212 Group 2 (12.3±6.8).
213

Discussion

In this study, pretreatment of mice with pulmonary fibrosis with nintedanib before administration of bleomycin, but not after administration of bleomycin, reduced the Ashcroft score, decreased collagen and TGF-β1 in BAL fluid was seen to be associated with a decrease in lung fibrosis. The pretreatment with both nintedanib and steroid exacerbated lung fibrosis in bleomycin-induced pulmonary fibrosis model mice compared with pretreatment with nintedanib only.

Bleomycin-induced lung fibrosis is a widely used animal model of pulmonary fibrosis [24]. Intratracheal administration of bleomycin induces acute alveolitis and interstitial inflammation, characterized by sequential recruitment of leucocytes in the first week, followed by fibrotic responses associated with fibroblast proliferation and synthesis of extracellular matrix in the second week [25]. Steroids have anti-inflammatory effects and nintedanib has anti-fibrotic effects [19, 26]. We demonstrated the effect of nintedanib, which has anti-fibrotic function, in mice with bleomycin-induced pulmonary fibrosis and then showed that bleomycin-induced pulmonary fibrosis was reduced by pretreatment with nintedanib before administration

230 of bleomycin, but not by treatment with nintedanib after administration of bleomycin.
231 The results were similar to those reported by Lutz et al. [27]. Fibroblasts are thought to
232 proliferate once activated and this cannot be suppressed. However, we demonstrated
233 that the concentration of TGF-β1 in BAL fluid decreased with the extent of lung fibrosis.
234 Conversely, FGF2 was not detected despite the presence of lung fibrosis. However, IHC
235 of lung tissues showed clearly that FGF2 was also expressed in fibroblasts. Depending
236 on the type, cytokines may not be detected in BAL. In addition, nintedanib had no
237 effect on PDGF-AA and VEGF-A in BAL fluid. PDGF and VEGF can be produced by
238 fibroblasts, but they are also produced in large amounts by endothelial cells, epithelial
239 cells, and macrophages [28, 29]. This suggests that even if both the activity and
240 proliferation of fibroblasts was suppressed by nintedanib, the cytokine concentration in
241 BAL was not affected. In addition, the treatment of nintedanib before and after
242 administration with bleomycin had no effect on the number of total cells, including
243 macrophages, lymphocytes, neutrophils and eosinophils in BAL (data not shown).
244 Clinically, it is desirable to begin administering nintedanib from the period when
245 fibroblast activity is as low as possible. Nintedanib, which is a TKI that acts via VEGF,

246 PDGF, and FGF receptors expressed on lung fibroblasts, may have suppressed
247 subsequent TGF-β1 and FGF2 production by directly suppressing fibroblast activity and
248 proliferation, regardless of inflammatory cells. In addition, the single treatment with
249 nintedanib before administration of bleomycin without the subsequent treatment with
250 nintedanib after administration of bleomycin did not suppress pulmonary fibrosis (data
251 not shown). This result indicates that treatment with nintedanib is optimal by continuous
252 administration.

253 When treating pulmonary fibrosis, it is important to control inflammation and
254 subsequent fibrosis. Steroids act on lymphocytes and macrophages to suppress
255 inflammatory cytokines and exert anti-inflammatory effects [30, 31]. However, there are
256 many negative reports about the effectiveness of steroids in ameliorating human IPF [32,
257 33]. In addition, studies in murine models showed that treatment with steroids did not
258 suppress lung fibrosis [34, 35]. The effectiveness of combining steroids with antifibrotic
259 drugs in human IPF is also unclear. For this reason, we analyzed the effectiveness of a
260 human antifibrotic drug and steroid in similar amounts using the bleomycin mouse
261 model. However, the results showed that the combinations of various nintedanib doses

and steroid could not suppress pulmonary fibrosis. Thus, pulmonary fibrosis aggravated by the single-agent administration of nintedanib was observed to be aggravated by the combined use of both agents after the administration of bleomycin, regardless of pretreatment with steroid. These findings indicate that this combined use after the induction of inflammation may not inhibit pulmonary fibrosis. Although the detailed mechanism is unknown, it is considered that the steroid may reduce the affinity of nintedanib for fibroblasts or this may be reduced by interaction between the drugs. In addition, although the dose of steroid was relatively high in this study, the effect on pulmonary fibrosis may be affected by the administration of a small dose of steroid.

In conclusion, nintedanib may be useful when administered early in patients with pulmonary fibrosis. Further, clinically, it is considered that a combination of nintedanib and steroid should be used with sufficient caution from the viewpoint of effects on pulmonary fibrosis and side effects.

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Conflict of interest:

All authors have no conflict of interest.

Abbreviations:

IPF: idiopathic pulmonary fibrosis, IIPs: idiopathic interstitial pneumonias, TGF: transforming growth factor, EMT: epithelial-mesenchymal transition, FVC: forced vital capacity, TNF: tumor necrosis factor, TKI: tyrosine kinase inhibitors, VEGF: Vascular endothelial growth factor, PDGF: platelet-derived growth factor, FGF: fibroblast growth factor, MDD: minimum detectable dose, BAL: bronchoalveolar lavage, HE: hematoxylin and eosin, EM: Elastica-Masson

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Figure legends

Figure 1; Effects of treatment with nintedanib before and after administration of bleomycin in mice. Saline (bleomycin[-]) (Group 1) or bleomycin sulfate (bleomycin[+]; 5 mg/kg body weight (Groups 2, 3, and 4) was given by intratracheal administration to mice on D1. Nintedanib (3 mg/kg body weight) was given by transesophageal administration before (D0) (Group 3) or after (D2) (Group 4) intratracheal administration of bleomycin in mice. As a control, saline was given by transesophageal administration after (D2) (Group 2) intratracheal administration of bleomycin. Subsequently, nintedanib (Groups 1, 3, and 4) or saline (Group 2) was administered daily, twice a day from D2 to D6 and from D8 to D13. Nint; nintedanib, BLM; bleomycin, BALF; bronchoalveolar lavage fluid, ELISA; Enzyme-Linked Immuno Sorbent Assay.

391

392 Figure 2; Effects of treatment with both nintedanib and steroid before and after
393 administration of bleomycin in mice. Figure 2 shows the study protocols. Group 2, 3,
394 and 4 were administered bleomycin to induce pulmonary fibrosis. Saline (bleomycin[-])
395 (Group 1) or bleomycin sulfate (bleomycin[+]; 5 mg/kg body weight) (Group 2, 3, and
396 4) was given by intratracheal administration to mice on D1. Both nintedanib (3 mg/kg
397 body weight) and dexamethasone (0.1 mg/kg body weight) was given by
398 transesophageal administration before (D0) (Group 3) or after (D2) (Group 4)
399 intratracheal administration of bleomycin. As a control, saline was given by
400 transesophageal administration after (D2) (Group 2) intratracheal administration of
401 bleomycin. Subsequently, both nintedanib and dexamethasone (Groups 1, 3, and 4) or
402 saline (Group 2) were given daily, twice and once a day, respectively, from D2 to D6
403 and from D8 to D13. Nint; nintedanib, Dex; dexamethasone, BLM; bleomycin, BALF;
404 bronchoalveolar lavage fluid.

405

406

Figure 3; Histopathological analysis [A to I] and collagen concentrations [J], TGF-β1 [K], VEGF-A [L], and PDGF-AA [M] in BAL fluid on D15 to determine the effects of nintedanib before and after administration of bleomycin in mice. For histopathological analysis, lung sections were stained with HE [A, C, E, G] and EM [B, D, F, H]. Group1; [A], [B], Group2; [C], [D], Group3; [E], [F], Group4; [G], [H]. Scale bar: 100 μm. Quantification of lung fibrosis [I] in lung tissue by score. Concentrations (ug/ml) of collagen in BAL fluid [J]. P values indicate comparisons between each group. Data are means ± SD for each of 6 to 11 mice. NS, non-significance. N.D, not detected.

Figure 4; Immunostaining analysis (TGF-β1 [A], FGF2 [C], PDGF-AA [E], and VEGF-A [G]) on D15 after administration of bleomycin. Scale bar: 20 μm, or 50μm. As controls, immunostaining analysis of TGF-β1 [B], FGF2 [D], PDGF-AA [F], and VEGF [H] is shown as negative results in which primary antibody were absent.

Figure 5; Histopathological analysis [A to I] and collagen concentrations [J] in BAL fluid on D15 for the effects of both steroid and nintedanib before and after

423 administration of bleomycin. For histopathological analysis, lung sections were stained

424 with HE [A, C, E, G] and EM [B, D, F, H]. Group1; [A], [B], Group2; [C], [D],

425 Group3; [E], [F], Group4; [G], [H]. Scale bar: 100 μm. Quantification of lung fibrosis

426 [I] in lung tissue by score. Concentrations (ug/ml) of collagen in BAL fluid [J]. Data are

427 means ± SD for each of the 9 to 11 mice. NS, non-significant.