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Ponatinib ameliorates pulmonary fibrosis by suppressing TGF-β1/Smad3 pathway

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ABSTRACT

TGF-β1/Smad3 pathway plays a key role in the pathogenesis of idiopathic pulmonary fibrosis, including lung fibroblasts proliferation and epithelial cell aberrant activation. Ponatinib is a multi-targeted tyrosine-kinase inhibitor. However, whether Ponatinib has anti-fibrotic functions is unknown. In this study, the effects of Ponatinib on TGF-β1-mediated epithelial–mesenchymal transition (EMT) in A549 cells, on the proliferation of human lung fibroblasts (HLF-1), on the apoptosis of human type I alveolar epithelial cells (AT I) *in vitro*, and on bleomycin (BLM)-induced pulmonary fibrosis was investigated *in vivo*. Treatment with Ponatinib resulted in a reduction of EMT in A549 cells with a decrease in vimentin and p-Smad3, whereas an increase in E-cadherin. Apoptosis of AT I was attenuated with an increase in the Bcl-2/Bax ratio. HLF-1 proliferation was reduced with a decrease in PDGF-BB and FGF-2 expressions. Treatment with Ponatinib resulted in an amelioration of the BLM-induced pulmonary fibrosis in rats with reductions of the pathological score, collagen deposition, p-Smad3, α-SMA, PDGF-BB and FGF-2 expression. In summary, Ponatinib reversed the EMT, inhibited the apoptosis of AT I, as well as HLF-1 proliferation and prevented pulmonary fibrosis by suppressing the TGF-β1/Smad3 pathway.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, irreversible and lethal disease, especially at the phase of unpredictable acute exacerbation is often fatal. Up till now, the cause is unknown [1–4]. The pathogenesis of IPF is not completely clarified. The latest evidences indicate that the fibrotic response is driven by abnormally activated alveolar epithelial cells (AECs) which produce mediators that induce the formation of fibroblast and myofibroblast foci through the proliferation of resident mesenchymal cells, the attraction of circulating fibrocytes, and stimulation of the epithelial to mesenchymal transition (EMT). The fibroblast and myofibroblast foci secrete amounts of extracellular matrix (ECM), mainly the collagen, resulting in scarring and destruction of the lung architecture [5]. The signaling pathways were activated by several tyrosine kinase receptors which was involved the progression of lung fibrosis, suggesting that the inhibition of these aztyrosine kinase receptors slows the progression of IPF.

PDGF and FGF promote proliferation and migration in a variety of cell types [6]. Pirfenidone (formerly BIBF 1120) is a small molecule tyrosine-kinase inhibitor, targeting vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptor (PDGFR) being developed by Boehringer Ingelheim, and recently approved for treatment of idiopathic pulmonary fibrosis [7,8]. Ponatinib (AP24534), a novel tyrosine-kinase inhibitor, is targeting PDGFRα, VEGFR2, FGFR1 [9,10]which is an FDA-approved oral drug candidate which was developed by ARIAD Pharmaceuticals for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Up till now, no pharmacological activity of Ponatinib has been reported in experimental pulmonary fibrosis. Therefore, we investigated the effects of Ponatinib on experimental pulmonary fibrosis *in vitro* and *in vivo* and proposed a mechanism of action.

Abbreviations: α-SMA, alpha smooth muscle actin; AECs, alveolar epithelial cells; AP, Ponatinib; AT I, human type I alveolar epithelial cell line; BLM, bleomycin; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FGF-2, fibroblast growth factor; HE, hematoxylin and eosin; HLF-1, human lung fibroblast; Hyp, hydroxyproline; IPF, idiopathic pulmonary fibrosis; PDGF, platelet-derived growth factor.

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2. Materials and methods

2.1. Cell culture

Human lung fibroblasts (HLF-1), human type I alveolar epithelial cells (AT I) and human type II alveolar epithelial cells (A549 cell line) were purchased from the Cell Bank of the Chinese Academy of Sciences.

2.2. Ethics statement

Adult male Sprague–Dawley rats (body weight 190–210 g) were housed individually under a constant temperature (22 ± 2 °C) and humidity with a 12 h light/dark cycle and with free access to chow and water. All animal experimental procedures in this study were performed in accordance with the Committee on the Ethics of Animal Experiments of Binzhou Medical University (Permit Number: SCXK 20130012).

2.3. TGF-β1 reducing human alveolar epithelial to mesenchymal cell transition (EMT) in vitro

The A549 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% (v/v) fetal bovine serum, 100 kU/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified 5% CO_2. The cells were cultured at approximately 70% confluency and starved in the serum-free DMEM overnight. Ponatinib (Hydrochloric acid Ponatinib, purity >99.0%, CAS NO.: 1232836-25-7, provided by NCE biomedical company, Wuhan, PR China.) was treated at concentrations of 0, 0.1 and 0.3 μM or Smad3 inhibitor, SIS3 (Santa Cruz Biotechnology, sc-222318) with or without TGF-β1 (5 ng/ml, Sigma) at the same time for 48 h; then pictures were taken in five random fields (×400) under the inverted microscope.

2.4. Determination of apoptosis in human type I alveolar epithelial cells (AT I)

The A549 cell line was cultured at approximately 70% confluency and starved in the serum-free DMEM overnight, followed by addition of 5 ng/mL TGF-β1 for 48 h, then collected the supernatant. AT I cells were maintained in DMEM/F12 containing 10% (v/v) fetal bovine serum, 100 kU/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. When the cells were cultured at approximately 80% confluency, DMEM/F12 was replaced with the collected supernatant containing the different concentrations of Ponatinib (0, 0.1, 0.3 and 1 μM), and the cells were cultured for 4 h. The apoptotic cells were evaluated with an Annexin-V FITC apoptosis detection kit. The same method was applied to analyze Bcl-2, Bax and receptor for advanced glycation end-product (RAGE) expression.

2.5. HLF-1 proliferation assay

For the proliferation assays *in vitro*, HLF-1 was inoculated into 96-well (1×10^5 cells/well) flat bottom plates in triplicate with medium alone (control) or with medium containing different concentrations of Ponatinib (0, 0.1, 0.3 and 1 μM) or a FGF-2/PDGF inhibitor Suramin (50 μM, Sigma), with or without 5 ng/mL of TGF-β1. Cell proliferation was tested by the cell counting method and calculated as a ratio against the untreated cells.

2.6. Western blot analyses

The A549 cells and HLF-1 were cultured for 48 h, and then washed twice with ice cold phosphate buffered saline (PBS) and lysed in NP40 lysis buffer. An equal amount of cell proteins (40 μg) were separated by SDS-PAGE and analyzed with the specific antibodies to vimentin, E-cadherin, Smad3, p-Smad3, PDGF-BB, FGF-2 and β-actin. Data was normalized against that of the corresponding β-actin bands which were scanned and quantified with a Gel Doc 2000 (Bio-Rad Laboratories Ltd). The results were expressed as fold increase over the control.

2.7. Bleomycin (BLM)-induced pulmonary fibrosis model

Thirty Sprague–Dawley (SD) rats with an average weight of 150 g were acclimatized for 7 continuous days. The pulmonary fibrosis model was established except for the control animals. The model was induced according to the previous method [11]. The control rats received an equal volume of saline only. At day 21, the pulmonary fibrosis rats were randomly divided into two groups according to body weight: BLM-treated group and Ponatinib 1 mg/kg (Ponatinib-treated) group. The Ponatinib-treated animals were orally administered Ponatinib daily. The lung was removed at day 35, and the lungs were divided into two parts: one part was frozen in liquid nitrogen, and the other part was fixed in 10% formalin for further analysis.

2.8. Histopathological examination

The lungs were fixed in 10% formalin and embedded in paraffin, and then the sections were prepared to 4 μm in thickness and stained with hematoxylin and eosin (HE) and Masson's trichrome. The grades of interstitial fibrosis were analyzed according to a previous method [12]. The average score of five fields was examined and calculated as the fibrosis score of each animal. The percentage of fibrosis in the lungs were evaluated according to a previously method [13].

2.9. Immunohistochemistry

The sections were deparaffinized and rehydrated, and then treated in 0.01 M citric acid at 400 W in a microwave for 10 min. The endogenous peroxidase was inactivated with 5% H_2O_2 in methanol for 30 min in dark at room temperature. Next, the sections were sealed with serum cap for 30 min and incubated with a rabbit polyclonal anti-α-SMA for 16 h at 4 °C; they were then washed and incubated with anti-rabbit horseradish peroxidase-conjugated antibody for 60 min at 37 °C. The samples were observed under the light microscopy.

2.10. Measurement of hydroxyproline (Hyp)

The lung tissues were washed with saline and hydrolyzed with 6 mL/L hydrochloric acid at 100 °C for 5 h. The Hyp content was determined at 560 nm with p-dimethylaminobenzaldehyde and was expressed as milligrams per gram of the wet lung tissue.

2.11. Western blot analysis of lung tissue

The samples of lung were suspended in a buffer and lysed by homogenization, collected the supernatant for western blot analysis. The protein concentration was measured and equal amount of the proteins (35 μg) were separated and analyzed with the specific antibodies to α-SMA, PDGF-BB, FGF-2 and β-actin. The results were expressed as fold increase over lung tissue of the control animal.