Roles of eicosanoids in pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease characterized by fibroblast proliferation and excess deposition of extracellular matrix proteins in the lungs. As anti-inflammatory therapies such as steroids or immunosuppressive agents do not improve disease progression, targets to block fibrogenesis per se are needed. Mice lacking cytosolic phospholipase A2, a key enzyme triggering the production of arachidonic acid metabolites such as eicosanoids including prostanoids and leukotrienes, did not develop bleomycin-induced pulmonary fibrosis, indicating that arachidonic acid metabolites play an important role in the pathogenesis of pulmonary fibrosis. Previous reports indicated that leukotrienes have fibrogenic effects, and prostaglandins (PGs) E2 and I2 have antifibrogenic effects. We have reported that, using mice lacking each prostaglandin receptor, PGF2α and its receptor FP signaling facilitated bleomycin-induced pulmonary fibrosis through increased fibroblast proliferation and collagen deposition, independently of transforming growth factor (TGF)-β signaling. Furthermore, to evaluate the clinical significance of PGF2α in IPF, using the liquid chromatography-tandem mass spectrometry, the levels of 15-keto-dihydro PGF2α as well as PGF2α increased in the bronchoalveolar lavage fluid of patients with IPF to a greater extent than in patients with sarcoidosis. Thus, we have suggested PGF2α-FP signaling as a therapeutic target for IPF. Eicosanoids may be promising targets for novel treatments and management of IPF, in addition to TGF-β.

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Introduction
Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease characterized by fibroblast proliferation and excess deposition of collagen and other extracellular matrix (ECM) proteins in the lungs. It is usually lethal and is of unknown etiology. There are limited therapeutic options. As the currently used anti-inflammatory and immunosuppressive therapies do not improve disease progression, therapies that block fibrogenesis per se are needed. Modeling pulmonary fibrosis with bleomycin has been most fre-
Various eicosanoids are involved in pulmonary fibrosis

Eicosanoids are produced through the activation of cPLA₂, which cleaves phospholipids in response to stimuli and releases arachidonic acid. It is metabolized by cyclooxygenase (COX) to produce prostanoids and by 5-lipoxygenase (5-LO) to produce leukotrienes. Prostanoids include prostaglandin (PG) D₂, PGE₂, PGF₂α, PGI₂ and thromboxane (TX) A₂ acting at their cognate receptors which are designated as DP, EP1 — EP4, FP, IP and TP, respectively. Leukotrienes are synthesized through an intermediate product of LT₄, which is converted to LT₅, or cysteinyl leukotrienes (LTC₄, LTD₄, LTE₄). Prostanoids and leukotrienes are released outside of cells, and act at the sites due to their short half-life. Using liquid chromatography-tandem mass spectrometry, we systematically measured the amount of arachidonic acid metabolites in the lung, and found that various eicosanoids are produced in the bleomycin-induced pulmonary fibrosis model. In addition, as loss of cPLA₂ suppressed fibrotic process, it may play an important role in the pathogenesis of fibrogenesis.

In experiments using mice deficient in 5-LO or CysLT2 receptor (a low affinity receptor for cysteinyl leukotrienes), or treated with a LTβr receptor antagonist, bleomycin-induced inflammation and its subsequent fibrosis were reduced, suggesting that leukotrienes may exert a proinflammatory action and then contribute to fibrosis. Thus, leukotriene receptor antagonists and Zileuton, a 5-LO inhibitor, which are currently used or studied in asthma, have been investigated for use in pulmonary fibrosis in animal models and humans.

On the other hand, regarding prostanoids, in experiments using COX-2-deficient mice, pulmonary fibrosis worsened or did not change compared with that in wild-type (WT) mice. Conversely, using indomethacin (COX inhibitor) or COX-2 inhibitor, bleomycin-induced pulmonary fibrosis was reduced. These inconsistent results indicate that different prostanoids may play opposing roles in the pathogenesis of pulmonary fibrosis. Among them, the antifibrotic effects of PGE₂ have been most investigated. PGE₂ reduces fibroblast activity through diminished fibroblast proliferation, migration and collagen production, mainly through EP2 receptor-mediated elevations in cyclic adenosine monophosphate (cAMP). EP2 levels are diminished in fibroblasts after bleomycin treatment, which resulted in blunted cAMP responses and a reduced ability of PGE₂ to inhibit proliferation and collagen production in fibroblasts. This seems to be due to hypermethylation of the PGE receptor 2 gene promoter in fibroblasts from IPF patients or fibrotic mice, or deficiency in enzyme PTEN, which upregulates EP2 expression, in the fibrotic foci. Thus, signaling via inhibitory EP2 receptor diminished, and then fibroblasts in patients with IPF are refractory to PGE₂ inhibitory signaling.

Similarly, PGI receptor, IP-deficient mice showed exaggerated fibrosis, and, conversely, iloprost, a PGI₂ analogue, abrogated bleomycin-induced pulmonary fibrosis. Thus, PGE₂-EP2 and PGI₂-IP signaling have protective roles in fibrogenesis; however, prostanoids promoting fibrosis remain to be elucidated.

FP signaling promotes fibrosis without affecting inflammation

To reveal the roles of prostanoids in pulmonary fibrosis, we subjected mice deficient in DP, EP1-3, FP, IP and TP and administered by a selective EP4 antagonist to bleomycin-
induced pulmonary fibrosis, and found out that only mice deficient in FP (FP<sup>−/−</sup>) showed a reduced fibrotic response as assessed by hydroxyproline contents, histology and lung function, compared with WT mice<sup>9</sup>. Furthermore, to analyze whether attenuated fibrosis is related to reduced inflammation or not, we studied the inflammatory response by examining the number of and composition of cells in bronchoalveolar lavage fluid (BALF), and found that it did not differ between the FP<sup>−/−</sup> and WT mice on Day 7 when inflammatory response peaked after bleomycin administration. Thus, FP signaling may directly promote fibrosis without affecting peak inflammatory responses.

We then performed gene expression analysis of the lungs of FP<sup>−/−</sup> and WT mice on Days 0, 7 and 14 after bleomycin administration<sup>10</sup>. Based on cluster analysis, the lung of FP<sup>−/−</sup> mice revealed induction of inflammation-related genes similarly to WT mice, but the induction of fibrosis-related genes was suppressed, indicating that PGF<sub>2α</sub>-FP functions not in inflammation but in fibrosis, where it facilitates fibrogenesis by enhancing the expression of fibrosis-related genes.

**FP signaling promotes fibrosis independently of TGF-β pathway**

Furthermore, to analyze the effect of PGF<sub>2α</sub>-FP signaling on the TGF-β pathway, firstly, using FP<sup>−/−</sup> and WT mice after bleomycin treatment, we measured the content of active TGF-β1 levels in BALF, and then assessed Smad2 phosphorylation, a crucial intracellular step in TGF-β signal transduction, in the lung homogenate. There were no significant differences in the increased active TGF-β1 levels and Smad2 phosphorylation between FP<sup>−/−</sup> and WT mice after bleomycin treatment. Additionally, we administered TGF-β receptor I kinase inhibitor, SD-208, to FP<sup>−/−</sup> and WT mice, and then found that FP deficiency and inhibition of TGF-β signaling additively decreased bleomycin-induced fibrosis. Thus, PGF<sub>2α</sub>-FP signaling and TGF-β signaling work independently of each other in the development of fibrosis in mice.

Fibroblasts are the key cells producing collagen and ECM proteins in fibrogenesis. PGF<sub>2α</sub> increased collagen production of lung fibroblasts via FP through stimulating fibroblast proliferation and the activity of the collagen α2(I) promoter, additively to TGF-β<sup>6</sup>. In addition, collagen production induced by PGF<sub>2α</sub> was inhibited by treatment with not only AL8810 (FP antagonist)<sup>20</sup> but also Y-27632<sup>21</sup>, a specific Rho kinase inhibitor, in IMR cells, a human lung fibroblast cell line. This indicates that PGF<sub>2α</sub> mobilize signaling to induce collagen production through the Rho kinase pathway differently from TGF-β1 whose main intracellular signaling is through Smad (Fig.1).

**PGF<sub>2α</sub> and other lipid mediators may be a therapeutic target for fibrosis**

We demonstrated that PGF<sub>2α</sub>-FP signaling mediates pulmonary fibrosis additive to that induced by TGF-β <i>in vivo</i> and <i>in vitro</i>. While the median survival of IPF patients of 3-5 years is short, developing drugs targeted against fibrogenesis is urgent. Although therapy against TGF-β signaling would be most promising, other therapeutic targets are needed and blocking PGF<sub>2α</sub>-FP signaling, for example, by an FP antagonist may be one possible alternate approach for treating pulmonary fibrosis. Recently, similar effects of PGF<sub>2α</sub> on collagen synthesis through protein kinase C and Rho kinase independently of TGF-β1 were observed in cardiac fibroblasts, indicating PGF<sub>2α</sub>-FP signaling as a new therapeutic target for myocardial fibrosis<sup>22</sup>. Thus, more attention may be paid to this pathway for treating fibrosis in various organs.

cPLA2 yields eicosanoids as well as lysophospholipids. Among lysophospholipids, lysophosphatidic acid (LPA) and one of its receptors, LPA1, are implicated in the pathogenesis of pulmonary fibrosis<sup>23</sup>, and an LPA1 antagonist is now being investigated in clinical trials of IPF or systemic sclerosis skin fibrosis<sup>10</sup>.
Plasma PGF$_{2a}$ metabolite may be a promising candidate for a biomarker of IPF

The clinical course of IPF is highly variable, and there are a considerable number of patients who show rapid disease progression (rapid decliners). Therefore, sensitive biomarkers that can predict future outcomes of patients with IPF are needed in clinical practice. Endogenous PGF$_{2a}$ is swiftly degraded in various organs to 13,14-dihydro-15-keto PGF$_{2a}$ (15-keto-dihydro PGF$_{2a}$), a stable metabolite of PGF$_{2a}$, which has a longer half-life in the circulation and has been used as a reliable indicator of in vivo PGF$_{2a}$ biosynthesis. We found that, using the aforementioned liquid chromatography-tandem mass spectrometry, the levels of 15-keto-dihydro PGF$_{2a}$ as well as PGF$_{2a}$ increased in the BALF of patients with IPF to a greater extent than in patients with sarcoidosis. We further measured plasma concentrations of this metabolite in patients with IPF. We then found that the plasma concentration of 15-keto-dihydro PGF$_{2a}$ in IPF patients was higher than that in controls, and was significantly associated with mortality (unpublished data). Thus, plasma 15-keto-dihydro PGF$_{2a}$ may have a prognostic value in IPF and be useful in clinical management of patients, although further study would be needed. Although the source of PGF$_{2a}$ and its metabolite remains speculative, activated macrophages, type II alveolar epithelial cells, metaplastic epithelium, and contractile interstitial cells (a suspected precursor of myofibroblasts) may produce PGF$_{2a}$, causing increased levels of PGF$_{2a}$ and its metabolite in BALF in IPF patients, which probably also leak into the circulation. In addition, pulmonary arterial endothelium and/or smooth muscles may upregulate the COX-2 pathway through hypoxic stimulus and/or development of pulmonary hypertension seen in advanced IPF, which increases the plasma levels.

Conclusion

Although many drug targets have been proposed through an animal model of IPF and many clinical trials have been performed for novel treatments in patients with IPF, effective therapies have not yet been established and the prognosis of IPF remains poor. However, recently, the understanding of IPF has progressed, and pirfenidone, a novel drug for IPF, has been developed. Studies on novel signaling pathways and effective clinical trials have to be continued. Eicosanoids including PGs (PGE$_2$, PGF$_{2a}$, PGI$_2$) and LTs may be one area of promising targets for novel treatments and management, different from the classical models of IPF.

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Conflict of interests

Authors declare no conflict of interest.

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