Pharmacological Targeting of Protease-Activated Receptor 2 Affords Protection from Bleomycin-Induced Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis is the most devastating diffuse fibrosing lung disease that remains refractory to therapy. Despite increasing evidence that protease-activated receptor 2 (PAR-2) contributes to fibrosis, its importance in pulmonary fibrosis is under debate. We addressed whether PAR-2 deficiency persistently reduces bleomycin-induced pulmonary fibrosis or merely delays disease progression and whether pharmacological PAR-2 inhibition limits experimental pulmonary fibrosis. Bleomycin was instilled intranasally into wild-type or PAR-2–deficient mice in the presence/absence of a specific PAR-2 antagonist (P2pal-18S). Pulmonary fibrosis was consistently reduced in PAR-2–deficient mice throughout the fibrotic phase, as evident from reduced Ashcroft scores (29%) and hydroxyproline levels (26%) at d 28. Moreover, P2pal-18S inhibited PAR-2–induced profibrotic responses in both murine and primary human pulmonary fibroblasts (p < 0.05). Once daily treatment with P2pal-18S reduced the severity and extent of fibrotic lesions in lungs of bleomycin-treated wild-type mice but did not further reduce fibrosis in PAR-2–deficient mice. Importantly, P2pal-18S treatment starting even 7 d after the onset of fibrosis limits pulmonary fibrosis as effectively as when treatment was started together with bleomycin instillation. Overall, PAR-2 contributes to the progression of pulmonary fibrosis, and targeting PAR-2 may be a promising therapeutic strategy for treating pulmonary fibrosis.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a lethal diffuse fibrosing lung disease with a 5-year mortality rate greater than 50%, which exceeds many types of cancers (1–3). IPF corresponds histopathologically to “usual interstitial pneumonia” (UIP) and comprises a pattern of fibrosis with interstitial fibroblast proliferation, alveolar destruction and excessive extracellular matrix (ECM) synthesis and accumulation (4). Studies exploring the mechanisms that are crucially involved in the development of IPF identified several possible targets for therapeutic interventions. Among those, protease-activated receptors (PARs) are key candidates, as these receptors mediate the cellular effects of coagulation factors and play central roles in influencing inflammatory and fibrotic responses (5–7).

PARs are seven-transmembrane G protein–coupled receptors which are activated by proteolytic cleavage by serine proteases (8). PAR-2 is one of four members of the PAR family that is widely expressed in many different cell types such as (among others) fibroblasts and epithelial cells (9). Proteases such as trypsin, factor (F)VIIa, FXa, mast cell tryptase or matriptase cleave the N-terminal extracellular domain of PAR-2, thereby revealing a novel tethered ligand that binds to PAR-2 and activates its transmembrane signaling to intracellular G proteins (10–12). Importantly, activated PAR-2 mediates a number of pathophysiological pathways involved in
acute/chronic inflammatory and fibrotic diseases of the joints, skin, brain, lung and gastrointestinal tract (10,12–15). With respect to lung injury and pulmonary fibrosis, there is increasing evidence that PAR-2 is a critical contributor in the pathogenesis of IPF. Increased PAR-2 expression has been detected in the lungs of patients with IPF, and a recent study proposed that the PAR-2/tissue factor (TF)/FVIIa axis may contribute to the development and/or progression of IPF (16). This study provided in vitro evidence that FVIIa exerts profibrotic effects in human fibroblasts by specifically activating PAR-2. In line with this finding, the prototypical PAR-2 agonist taspase stimulates the growth of human lung fibroblasts and potentiates extracellular matrix production in a PAR-2–dependent manner (17). Moreover, PAR-2 expression significantly correlates with the extent of honeycombing (18), and PAR-2 activation has been shown to be highly relevant to the progression of pulmonary fibrosis in experimental animal models of bleomycin-induced injury. Indeed, genetic ablation of PAR-2 in mice affords protection from pulmonary fibrosis, as evident from a reduction in the extent and severity of fibrotic lesions and diminished collagen expression (19). In addition, treatment of pulmonary fibrosis–bearing rats with diallylsulfide or daidzein results in amelioration of collagen production through the involvement of PAR-2 (20,21). Notwithstanding the overwhelming amount of data supporting an important role of PAR-2 in pulmonary fibrosis, some controversy has also emerged over the topic. Indeed, using a similar model of bleomycin-induced pulmonary fibrosis as in the study mentioned above, edema and hydroxyproline levels were not different between wild-type and PAR-2–deficient mice in a study by Su and colleagues (22). Also, a recent study showed that protein and mRNA expression levels of PAR-2 in IPF patients were not different from those of controls (23), and it has been claimed that it is doubtful whether blocking PAR-2 would serve as an effective treatment strategy for IPF (24). It is thus fair to state that, despite intriguing data supporting a role for PAR-2 in pulmonary fibrosis, its potential clinical relevance remains controversial. In the present study, we addressed the controversy by first assessing whether PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis or merely delays disease progression. We reaffirmed the importance of PAR-2 and subsequently evaluated the efficacy of pharmacological PAR-2 inhibition in pulmonary fibrosis.

MATERIALS AND METHODS

Cells and Reagents

Mouse embryonic NIH/3T3 fibroblasts (American Type Culture Collection; ATCC CRL-1658) and primary human lung fibroblasts (derived from pulmonary control and IPF patient explants as described in [25]; provided by INSERM U1152) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were grown at 37°C in an atmosphere of 5% CO2. Unless indicated otherwise, cells were washed twice with PBS and serum starved for 4 h before stimulation. Human platelets were donated by a healthy volunteer and isolated as described before (26). Thrombin and trypsin were from Sigma-Aldrich, whereas P2pal-18S (PAR-2 inhibitor [27]; palmitateRSSAMDENSEKKRKSAIK-NH2) was from GL Biochem Ltd.

Calcium Assay

Calcium signaling responses were analyzed using the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen) following the manufacturer’s instructions. Cells were preincubated with or without 10 μmol/L P2pal-18S and then challenged with PAR-1 agonist. Ca2+ flux was monitored for the indicated time points on a Bio-Tek HT Multi-Detection Microplate Reader (Winooski).

Western Blot

Western blots were performed essentially as described before (19). In brief, cells were lysed in Laemmli lysis buffer and the lysates were incubated for 5 min at 95°C. Afterward, protein samples were separated by 10% SDS gel electrophoresis and transferred to a PVDF membrane (Millipore). Membranes were blocked for 1 h in 4% milk in TBST and incubated overnight with monoclonal antibodies against α-smooth muscle actin (α-SMA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or collagen (all Santa Cruz Biotechnology, Inc.) at 4°C. All secondary antibodies were horseradish peroxidase (HRP)-conjugated from DakoCytomation and diluted according to the manufacturer’s instructions. Blots were imaged using Lumilight plus ECL substrate from Roche on an ImageQuant LAS 4000 bioluminescent imager from GE Healthcare. For quantification, densitometry was performed using ImageJ.

Animal Model of Pulmonary Fibrosis

Wild-type C57Bl/6 mice were purchased from Charles River. PAR-2–deficient (PAR-2−/−) C57Bl/6 mice were originally provided by Jackson Laboratories and bred at the animal care facility of the Academic Medical Center. All procedures were performed on 10-wk-old mice in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals of the Academic Medical Center. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center. Bleomycin (Sigma-Aldrich) was administered by intranasal instillation (1 mg/kg body weight in 45 μL saline) under anesthesia. In the PAR-2 pepducin dose-finding experiment, animals were intranasally instilled with 2.5 or 10 mg/kg P2pal-18S half an hour before bleomycin administration and subsequently once daily until the end of the experiment. In the delayed treatment experiment, mice were treated once daily with 2.5 mg/kg pepducin starting 1, 3 or 7 d after bleomycin instillation. In both experiments, 6% DMSO in saline was administered as a solvent control. Unless stated otherwise, mice were killed 14 d after bleomycin instillation, after which one lung was taken for histological analysis and one was homogenized.
Bronchoalveolar Lavage
At 14 d after bleomycin instillation, the lungs were lavaged three times with 0.3 mL of saline by a 22-gauge Abbocath-Tcatheter into the trachea via a midline incision before animals were killed. The bronchoalveolar lavage fluid (BALF) was centrifuged, and the supernatant was frozen at –70°C until use.

TGF-β ELISA
Transforming growth factor β-1 (TGF-β1) was measured with the Mouse TGF-β1 DuoSet kit (R&D systems) per the manufacturer’s instructions.

Hydroxyproline Assay
Hydroxyproline analysis was performed by the hydroxyproline assay kit per the manufacturer’s instructions (Sigma-Aldrich) and as described before (28).

Histological Analysis
Histological examination was performed essentially as described before (19). Briefly, the excised lung was fixed in formalin and embedded in paraffin, and then 4-μm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) according to routine procedures. In H&E staining, the severity of fibrosis was assessed according to the Ashcroft scoring system (29) using a 100× magnification. Two independent observers were blinded to the treatment group, and an average of 10 fields of each lung section was selected and scored. The average Ashcroft score was calculated by averaging the individual field scores.

Statistics
Statistical analyses were conducted using GraphPad Prism version 5.00 (GraphPad software). Data were expressed as means ± SEM. Comparisons between two conditions were analyzed using two-tailed unpaired t tests when the data were normally distributed, otherwise Mann-Whitney analysis was performed. P values of less than 0.05 were considered significant.

RESULTS
PAR-2 Deficiency Attenuates Bleomycin-Induced Pulmonary Fibrosis
We previously showed that PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis when we analyzed mice 14 d after bleomycin instillation (19,30). To determine whether PAR-2 indeed drives the progression of pulmonary fibrosis or whether its absence would merely delay the onset of fibrosis, we compared lungs from wild-type and PAR-2-deficient mice at d 28 and 48 after the instillation of bleomycin. As shown in Figure 1A, PAR-2-deficient mice showed some degree of resolution by d 48 (Figures 1C, D). Quantification of the bleomycin-induced histological changes in wild-type and PAR-2-deficient mice using the Ashcroft score shows that PAR-2-deficient mice develop less severe fibrotic lesions at d 28 than wild-type mice (Figures 1E, F). These observations are also supported by hydroxyproline levels indicating that collagen deposition is significantly reduced in PAR-2-deficient lungs at d 28 (Figures 1G, H). Overall, these results show that PAR-2 deficiency indeed provides protection against bleomycin-induced pulmonary fibrosis throughout the fibrotic stage and does not merely delay disease onset.

P2pal-18S Effectively Inhibits PAR-2-Mediated Profibrotic Responses in Fibroblasts
Next, we determined the specificity of the observed effects in PAR-2-deficient mice by assessing whether pharmacological PAR-2 inhibition by P2pal-18S would reduce lung fibrosis as well. Before assessing the in vivo effect of P2pal-
We first determined its specificity for blocking PAR-2–dependent fibrotic responses in vitro. Although the specificity of P2pal-18S has been tested previously (27), we here used human platelets (not expressing PAR-2) to confirm that P2pal-18S targets only PAR-2 and does not interact with other PARs. As shown in Figure 2A, PAR-1 activation by thrombin induced Ca2+ influxes that peaked within 1 min after stimulation, whereas pretreatment with 10 μmol/L P2pal-18S did not interfere with this response.

We next assessed whether P2pal-18S inhibits PAR-2–driven profibrotic responses in fibroblasts. We show that trypsin induced fibroblast differentiation, as evident from increased α-SMA expression and collagen production, compared with vehicle-treated cells. Trypsin-induced α-SMA and collagen expression were clearly downregulated by the treatment with 10 μmol/L P2pal-18S (Figure 2B). Bleomycin administration is known to increase PAR-2 agonist expression in BALF, which may lead to fibroblast differentiation or ECM synthesis (19, 31). We therefore next assessed the inhibitory effect of P2pal-18S against BALF-induced profibrotic effects on murine fibroblasts. NIH3T3 cells seeded in BALF of wild-type mice treated with bleomycin showed increased expression of α-SMA and collagen compared with cells seeded in BALF derived from saline-treated mice, whereas preincubation with P2pal-18S partially decreased this effect (Figure 2C). Finally, we determined whether...
P2pal-18S also limits PAR-2–induced fibrotic responses on primary human lung fibroblasts. We stimulated fibroblasts isolated from nonfibrotic controls and IPF patients with trypsin in the absence or presence of P2pal-18S. IPF-derived fibroblasts exhibited high α-SMA and collagen expression persistently (Figures 2D and E showing three independent fibroblast isolations), whereas control fibroblasts showed increased α-SMA and collagen expression only after treatment with trypsin. The expression of α-SMA and collagen were reduced by P2pal-18S in both control and IPF fibroblasts (Figures 2F, G). Importantly, P2pal-18S does not affect unstimulated IPF fibroblasts, indicating that the inhibitory effect is specific to PAR-2 stimulation (Supplementary Figure S1). Overall these in vitro data show that P2pal-18S effectively antagonizes PAR-2-mediated profibrotic effects in both murine and human fibroblasts.

**P2pal-18S Limits the Development of Pulmonary Fibrosis in the Bleomycin Model**

After having shown that P2pal-18S inhibits PAR-2–induced fibroproliferative responses in fibroblasts, we set out to examine whether P2pal-18S would effectively limit fibrosis in the bleomycin model. To this end, mice were intranasally instilled with different concentrations of P2pal-18S (based on previous experiments showing that a similar pepducin-based inhibitor [P1pal-12; PAR-1 inhibitor] was effective at doses of 2.5 and 10 mg/kg but not at a dose of 0.5 mg/kg [28]), after which the extent and severity of fibrosis was determined at d 14. As shown in Figure 3A, bleomycin instillation resulted in extensive fibrotic changes in lungs treated with saline or bleomycin instillation in saline treated mice (A) and mice treated with 2.5 mg/kg (B) or 10 mg/kg P2pal-18S (C). Scale bar, 200 μm; (D) Quantification of pulmonary fibrosis in control and P2pal-18S-treated mice using the Ashcroft score. Data are expressed as mean ± SEM (n = 8 per group). Total collagen content (E) and TGF-β1 levels (F) in lung homogenates of the different groups of mice obtained 14 d after saline or bleomycin instillation. Data are expressed as mean ± SEM (n = 8 per group; *P < 0.05; **P < 0.01).

Delayed Treatment with P2pal-18S Effectively Limits Pulmonary Fibrosis Progression in the Bleomycin Model

After having established that long-term treatment with P2pal-18S effectively limits bleomycin-induced pulmonary fibrosis, we assessed whether preventive and therapeutic modes of administration would still be effective. The bleomycin model is divided into two phases, namely the inflammatory phase (up to 7 d after bleomycin challenge) and the fibrosing phase (after 7 d) (33). P2pal-18S treatment (2.5 mg/kg) was consequently started for either 1, 3 (preventive treatment; concurrent with the inflammatory phase) or 7 d (therapeutic treatments; upon the establishment of lung fibrosis) after bleomycin instillation and continued once daily till the end of the experiment. As shown in Figures 4A–C, histological analysis of lung slides from bleomycin-instilled mice not treated with P2pal-18S again showed severe fibrotic lesions (about a five-fold increase compared with lungs of non–bleomycin-instilled controls). Treatment with P2pal-18S in the preventive mode strongly inhibited bleomycin-induced pulmonary fibrosis, as evident...
from lower Ashcroft scores (Figure 4G) and reduced collagen accumulation (Figure 4H). Interestingly, similar findings were obtained when P2pal-18S was administered in the therapeutic mode (compare Figures 4A and D). Indeed, P2pal-18S treatment starting on d 7 reduced the bleomycin-induced increase in Ashcroft score (although not statistically significant) and collagen deposition (Figures 4G, H). To corroborate the specificity of P2pal-18S, we analyzed the effect of P2pal-18S treatment (2.5 mg/kg, starting concurrently with bleomycin instillation) in bleomycin-treated PAR-2–deficient mice. As shown in Figures 4E and F, PAR-2–deficient mice developed less fibrosis than wild-type mice. More importantly, P2pal-18S treatment did not further reduce fibrosis and collagen deposition in the PAR-2–deficient mice (Figures 4I, J), suggesting that P2pal-18S limits pulmonary fibrosis by specifically targeting PAR-2.

DISCUSSION

IPF is a progressive lung disorder for which very few therapeutic options are available (1,3). It is often suggested as an uncontrolled wound healing response, in which multiple effectors are tightly involved. PAR-2, as a critical receptor that orchestrates a diverse range of signaling pathways, is suggested to play an important role in mediating profibrotic effects in fibroblasts and in preclinical experimental animal models (7,19,20,21,34–36). Importantly however, the relevance of PAR-2 in pulmonary fibrosis has recently been debated, and the actual role of PAR-2 in pulmonary fibrosis is controversial. In the present study, we aimed to address the controversy and provide several lines of evidence to support the crucial role of PAR-2 in the progression of pulmonary fibrosis. Bleomycin-induced pulmonary fibrosis is stably formed at around d 14, which is consequently frequently used as an endpoint in the literature (19,28,37). However, the fibrotic stage induced by bleomycin peaks at around 3–4 wks and the reduction in fibrosis observed in PAR-2–deficient mice at d 14 (19,30) may thus indicate only a delay in the onset of fibrosis instead of reduced progression. To address this concern, we increased the follow-up time and showed that the severity of fibrosis as well as collagen accumulation are significantly reduced in PAR-2–deficient mice compared with wild-type mice 28 d after bleomycin instillation. Furthermore, both wild-type and PAR-2–deficient mice enter the resolution phase of fibrosis by d 48, indicating the peak of fibrosis has passed. By using different endpoints of the murine bleomycin model, we provide evidence that PAR-2 deficiency provides persistent protection throughout the development of pulmonary fibrosis, suggesting PAR-2 is indeed an interesting target for therapeutic intervention. P2pal-18S is a cell-penetrating lipopeptide “pepducin” antagonist of PAR-2 that efficiently blocks PAR-2–driven profibrotic responses in vitro. Indeed, preincubation of murine fibroblasts with P2pal-18S blocks trypsin-induced extracellular signal-regulated kinases (ERK) phosphorylation, reduces proliferation and mi-
istration (30) and diminishes α-SMA and collagen expression (Figure 2). Moreover, P2pal-18S significantly inhibits collagen expression not only in control fibroblasts but also in IPF-derived human fibroblasts. IPF-derived human fibroblasts actually exhibit a more differentiated and fibrotic myofibroblast phenotype, as evident from elevated basal expression levels of both α-SMA and collagen, which may explain the reduced responsiveness to trypsin stimulation compared with control fibroblasts. As opposed to complete inhibition of trypsin-induced signaling, P2pal-18S only partly blocked ECM deposition induced by BALF from bleomycin-treated wild-type mice. This suggests that BALF partially exerts profibrotic effects through PAR-2 activation but that BALF also contains additional profibrotic components. Overall, these data show that P2pal-18S efficiently blocks PAR-2–dependent fibrotic responses in vitro. In line with a potential therapeutic role of PAR-2 in pulmonary fibrosis, we show for the first time that P2pal-18S limits bleomycin-induced pulmonary fibrosis even when administered at the fibrotic phase. As elegantly reviewed, bleomycin administration to rodents results in direct damage to alveolar epithelial cells, which is followed by an inflammatory response within the first week (33). Once alveolar inflammatory cells are cleared, activated fibroblasts start to proliferate and to synthesize ECM, which can already be visualized at d 7 after bleomycin instillation. Treatments during the first 7 d after bleomycin administration are consequently considered “preventive,” whereas treatments starting at d 7 are considered “therapeutic” (38). After showing that once daily P2pal-18S treatment (2.5 mg/kg) during the 14 d of the experiment significantly limited bleomycin-induced pulmonary fibrosis, we next showed that administration of P2pal-18S also effectively decreases the histological grade of pulmonary fibrosis and collagen contents in both a preventive (1- and 3-d delayed treatment) and therapeutic (7-d delayed treatment) mode. Importantly, the protective effect afforded by PAR-2 deficiency against bleomycin-induced pulmonary fibrosis was nearly identical to that observed in wild-type mice treated with P2pal-18S. In PAR-2–deficient mice as well as in P2pal-18S–treated mice, the severity of the fibrotic lesions assessed by the Ashcroft score was decreased by around 1 point. In line with this result, quantitative measurement of collagen deposition in the lungs was reduced by about 34% in PAR-2–deficient mice and 29% in P2pal-18S treated mice. Importantly, the antifibrotic effect of P2pal-18S was lost in PAR-2−/− mice, demonstrating that P2pal-18S is highly specific for PAR-2 in vivo. This is in accordance with a previous study (27) showing that P2pal-18S significantly reduces mouse paw edema and inflammation in wild-type but not PAR-2–deficient mice. Moreover, our in vitro data show that P2pal-18S does not interfere with thrombin-induced calcium fluxes in human platelets. As platelets respond to thrombin through PAR-1 and/or PAR-4 (39), these data indicate that P2pal-18S does not have cross-reactivity with other PARs.

CONCLUSION

Together, our data indicate that P2pal-18S provides effective and specific pharmacologic inhibition of PAR-2 in an animal model of pulmonary fibrosis and that inhibition of PAR-2 may thus be a promising therapeutic strategy for treating pulmonary fibrosis, although future clinical studies are needed to confirm this notion. It is noteworthy that, similarly to what has been observed for targeting PAR-1 in pulmonary fibrosis (28), P2pal-18S at a dose of 2.5 mg/kg seemed to be more effective in reducing fibrosis than a high dose of 10 mg/kg. Although we do not have a definite explanation for this finding, it is tempting to speculate that the phenomeon may be due to the fact that PAR-2, again similarly to PAR-1, acts both as a pro- and antiinflammatory receptor in the respiratory system. In cultured human epithelial cells, activation of PAR-2 causes release of proinflammatory cytokines such as interleukin (IL)-8 (40). PAR-2 stimulation is also required for serralysin to activate the critical transcription factors for host inflammatory responses (41). On the other hand, PAR-2 activation enhances LPS-induced expression of the antiinflammatory cytokines while suppressing gene expression of proinflammatory cytokines (42) and PAR-2–toll-like receptor (TLR) signaling integration drives “customized” inflammatory responses (43). Therefore, the extent of PAR-2 inhibition may affect the balance of its pro- and antiinflammatory properties. Alternatively, the slight differences observed between the 2.5- and 10-mg doses may simply be attributed to the variability within the analysis rather than any definable biological phenomenon, suggesting a dose of 2.5 mg/kg did already maximally block PAR-2 signaling. Pharmacological PAR-2 inhibition may not just benefit pulmonary fibrosis, due to the fact that PAR-2 influences a large range of pathophysiological pathways, and indeed PAR-2 deficiency affords protection from, among others, renal fibrosis (36), heart failure (44) and pulmonary hypertension (45). In line with these data, PAR-2 inhibition reduced, again among others, pulmonary hypertension, acute biliary pancreatitis and osteoarthritis (46,47). It is thus tempting to speculate that P2pal-18S may have an impact on a plethora of other (PAR-2 dependent) disorders, although this tantalizing hypothesis needs to be addressed experimentally. In summary, our data endorse the importance of PAR-2 in mediating profibrotic effects and identify the PAR-2 antagonist P2pal-18S as an effective inhibitor of bleomycin-induced pulmonary fibrosis. Thus, targeting PAR-2 signaling with P2pal-18S may offer a novel therapeutic option for patients with pulmonary fibrosis.

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DISCLOSURE
The authors declare they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.