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Protease-Activated Receptor-2 Induces Myofibroblast Differentiation and Tissue Factor Up-Regulation during Bleomycin-Induced Lung Injury

Potential Role in Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis constitutes the most devastating form of fibrotic lung disorders and remains refractory to current therapies. The coagulation cascade is frequently activated during pulmonary fibrosis, but this observation has so far resisted a mechanistic explanation. Recent data suggest that protease-activated receptor (PAR)-2, a receptor activated by (among others) coagulation factor (F)Xa, plays a key role in fibrotic disease; consequently, we assessed the role of PAR-2 in the development of pulmonary fibrosis in this study. We show that PAR-2 is up-regulated in the lungs of patients with idiopathic pulmonary fibrosis and that bronchoalveolar lavage fluid from these patients displays increased procoagulant activity that triggers fibroblast survival. Using a bleomycin model of pulmonary fibrosis, we show that bleomycin induces PAR-2 expression, as well as both myofibroblast differentiation and collagen synthesis. In PAR-2−/− mice, both the extent and severity of fibrotic lesions are reduced, whereas myofibroblast differentiation is diminished and collagen expression is decreased. Moreover, fibrin deposition in the lungs of fibrotic PAR-2−/− mice is reduced compared with wild-type mice due to differential tissue factor expression in response to bleomycin. Taken together, these results suggest an important role for PAR-2 in the development of pulmonary fibrosis, and the inhibition of the PAR-2-coagulation axis may provide a novel therapeutic approach to treat this devastating disease. ***(Am J Pathol 2010, 177:2753–2764; DOI: 10.2353/ajpath.2010.091107)***

Idiopathic pulmonary fibrosis (IPF) constitutes the most devastating form of fibrotic idiopathic interstitial pneumonias.[1,2] It generally evolves toward a destructive and irreversible fibrosis, responsible for respiratory failure. The median survival is 2 to 5 years.[3,4] Although substantial efforts led to considerable improvement in diagnosing the diseases and predicting the outcome, fibrotic lung diseases remain generally refractory to current available pharmacological therapies.[4,5] Interestingly, coagulation cascade activation is frequently observed in lungs of patients with fibrotic lung diseases. Enhanced expression of tissue factor (TF) and intra-alveolar fibrin deposition were demonstrated in lung biopsy specimens from patients with pulmonary fibrosis.[6–9] Procoagulant activity was demonstrated in bronchoalveolar lavage fluid (BALF) of patients with various forms of pulmonary fibrosis. More-

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over, local procoagulant activity was demonstrated in patients with acute lung injury.[6,8–10] The mechanistic link between coagulation activation and pulmonary fibrosis, however, remains elusive. Recent data suggest that protease-activated receptor 2 (PAR-2), a cellular receptor activated by (among others) coagulation factor (F)Xa, is an important player in fibrotic disease. PAR-2 triggers fibroproliferative responses *in vitro*[11–13] and is highly expressed in fibrotic disorders.[12] This study explores the role of blood coagulation factor driven PAR-2 activation in pulmonary fibrosis. We show that PAR-2 is up-regulated in the lung of IPF patients and that BALF of these patients displays increased procoagulant activity and enhances fibroblast survival. Using a mouse model of bleomycin-induced lung fibrosis, we show that PAR-2 deficiency is protective. Moreover, BALF of bleomycin-treated PAR-2 deficient mice less effectively induced fibroblast survival compared to BALF of wild-type treated mice; and bleomycin induced less coagulation activation in PAR-2 deficient mice. Taken together, PAR-2 seems to contribute to the progression of pulmonary fibrosis, and inhibition of the PAR-2-coagulation axis may be clinically relevant.

Materials and Methods

Patients

Eight patients with biopsy-proven IPF (mean age 67 ± 6 yrs) were included in this study; IPF was defined according to international guidelines (American Thoracic Society/European Respiratory Society[14]). Pathology specimens were judged according to the criteria described by Katzenstein and Myers.[1] In the IPF patients, bronchoalveolar lavage was performed as part of the routine workup; the procedure of bronchoalveolar lavage was previously described.[15] BALF from nine healthy volunteers (mean age 32 ± 8 yrs) who had participated in a previously reported study[15] served as controls. From five patients, enough BALF was available to study *in vitro* stimulation of human lung fibroblast. Control BALF was obtained from healthy volunteers. The study was approved by the Institutional Scientific and Ethics committees. Written informed consent was obtained from all subjects.

Assays

The levels of thrombin-antithrombin and that of soluble TF were measured in the BALF of IPF patients and controls using specific commercially available enzyme-linked immunosorbent assays (thrombin–antithrombin complex: Behringwerke AG, Marburg, Germany; soluble TF: American Diagnostics, Greenwich, CT) following the manufacturer's instructions.

Animals

Ten-week-old wild-type C57Bl/6 mice were purchased from Charles River (Someren, the Netherlands). PAR-2 deficient (PAR-2−/−) C57Bl/6 mice were originally provided by Jackson Laboratories (Maine) and bred at the animal care facility of the Academic Medical Center. All mice were maintained according to institutional guidelines. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. The Animal Care and Use Committee of the Academic Medical Center (Amsterdam, the Netherlands) approved all experiments. In each experimental group, eight mice were used. For baseline characteristics, four mice per genotype were used.

Experimental Model of Pulmonary Fibrosis

Bleomycin sulfate (1 mg/kg body weight in 45 μl of saline as described in[16]; Sigma, St-Louis, MO) was administrated by a single intratracheal injection under anesthesia. Control animals received an intratracheal saline injection. Mice were sacrificed 7 and 14 days after bleomycin instillation.

BALF

Bronchoalveolar lavage was performed by instilling four times 0.3 ml aliquots of saline by a 22-gauge Abbocath-T catheter into the trachea via a midline incision.[17]

Total Cell Number and Differential Cell Count

Total BALF cell numbers were assessed with a Burker-Turk hemocytometer (Emergo, Landsmeer, The Netherlands). Briefly, cells were pelleted by centrifugation, then resuspended in PBS and counted according to the manufacturer's recommendations. Differential BALF cell counts were performed by cytospins prepared by centrifuging 100 μl of BALF suspensions containing 200,000 cells/ml. Slides were air dried and stained with Giemsa. Cells were identified as macrophages/monocytes, neutrophils, or lymphocytes, and data were expressed in absolute numbers.

Histopathological Assessment of Pulmonary Fibrosis

The right lung of each animal was excised 7 or 14 days after bleomycin or saline treatment. Following fixation, entire mouse lungs were embedded in paraffin. Four-micron thick sections were stained with H&E, Masson's trichrome, and Sirius red, according to routine procedures. In H&E-stained lung sections, three systems were used to assess fibrosis (see Table 1[18,19]). For each scoring, observers blinded to the treatment group and genotype examined between 4 and 16 fields in all lung lobes (depending on the size and the homogeneity of the histological changes) using light microscopy (×200 magnification). Fields were examined to cover each entire lobe and were discarded if nonrepresentative areas such as airway lumen occupied >50% of the field of view. The mean score for each lobe was expressed as the average of scores determined in each field. The first scoring sys-