Absence of Proteinase-Activated Receptor-1 Signaling Affords Protection from Bleomycin-Induced Lung Inflammation and Fibrosis

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Activation of the coagulation cascade is commonly observed in the lungs of patients with both acute and chronic inflammatory and fibrotic lung disorders, as well as in animal models of these disorders. The aim of this study was to examine the contribution of the major thrombin receptor, proteinase-activated receptor-1 (PAR-1), during the acute inflammatory and chronic fibrotic phases of lung injury induced by intratracheal instillation of bleomycin in mice. Inflammatory cell recruitment and increases in bronchoalveolar lavage fluid (BALF) protein were attenuated by $56 \pm 10\%$ ($P < 0.05$) and $53 \pm 12\%$ ($P < 0.05$), respectively, in PAR-1-deficient (PAR-1^{-/-}) mice compared with wild-type (WT) mice. PAR-1^{-/-} mice were also protected from bleomycin-induced pulmonary fibrosis with total lung collagen accumulation reduced by $59 \pm 5\%$ ($P < 0.05$). The protection afforded by PAR-1 deficiency was accompanied by significant reductions in pulmonary levels of the potent PAR-1-inducible proinflammatory and profibrotic mediators, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor- β -1 (TGF- β), and connective tissue growth factor/fibroblast-inducible secreted protein-12 (CTGF/FISP12). In addition, PAR-1 was highly expressed in inflammatory and fibroproliferative lesions in lung sections obtained from patients with fibrotic lung disease. These data show for the first time that PAR-1 signaling plays a key role in experimentally induced lung injury, and they further identify PAR-1 as one of the critical receptors involved in orchestrating the interplay between coagulation, inflammation, and remodeling in response to tissue injury. (*Am J Pathol* 2005, 166:1353–1365)

There is accumulating evidence that the intra- and extravascular activation of coagulation proteinases contributes to inflammation and fibrosis in response to tissue injury in a number of organs, including the lung.¹ Extravascular intra-alveolar accumulation of fibrin, often evident as hyaline membranes, is commonly observed in acute lung injury, in the acute respiratory distress syndrome,² and in patients with pulmonary fibrosis,³ in which rapid fibroproliferation and matrix synthesis can lead to the development of extensive fibrotic lesions. Excessive procoagulant activity and intra-alveolar fibrin deposition observed in these conditions are thought to arise from an imbalance between locally produced pro- and anti-coagulant factors, in combination with leakage of plasma proteins (including fibrinogen) into the alveolar space. In the normal lung, the alveolar hemostatic balance is generally antithrombotic and profibrinolytic. However, in acute lung injury and in chronic lung diseases such as pulmonary fibrosis, this balance appears to be shifted with evidence of increased procoagulant (predominantly tissue factor/factor VII/VIIa complexes) and decreased fibrinolytic activity.^{4,5}

In addition to their critical role in blood coagulation, it is now well recognized that coagulation proteinases exert potent proinflammatory and profibrotic effects *via* activation of proteinase-activated receptors (PARs).⁶ The PARs currently comprise four members, PAR-1 to PAR-4, which are activated after the unmasking of a tethered ligand by limited proteolysis. Collectively, the proteinases of the coagulation cascade can target all four family members. Thrombin is considered to be a major activator of PAR-1, PAR-3, and PAR-4; whereas factor Xa, either on its own or as part of the tissue factor-factor VIIa-factor Xa ternary complex, can activate either PAR-1 or PAR-2, depending on cell type.⁷ Coagulation proteinases, including throm-

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bin and factor Xa, are elevated in bronchoalveolar lavage fluid (BALF) obtained from patients with acute and chronic forms of fibrosing and inflammatory lung disease.^{8,9,2} Procoagulant activity and thrombin levels are also increased in mouse and rat BALF after bleomycin-induced lung injury;^{10,11} and furthermore, immunohistochemical localization studies performed in this model have revealed prominent staining for thrombin and its receptor, particularly on infiltrating macrophages and fibroblasts, in the extravascular compartment.¹¹ Finally, we and others^{11,12} have shown that modulation of thrombin levels within the alveolar compartment attenuates lung inflammation and fibrosis in response to bleomycin-induced lung injury in experimental animals.

At present, the contributions of the procoagulant versus the PAR-mediated cellular effects after bleomycin-induced lung injury remain uncertain. In support of the hypothesis that fibrin may contribute to progression of fibrosis, experimental strategies that favor fibrin clearance, such as deletion of the PAI-1 gene¹³ or overexpression of urokinase-type plasminogen activator (uPA),¹⁴ have been associated with an attenuation in fibrosis after bleomycin instillation. However, studies reporting that fibrinogen knockout mice were not protected from bleomycin-induced lung fibrosis^{13,15} have questioned the importance of fibrin and its associated proinflammatory and profibrotic effects in this model.

In this study, we focused on the potential role of the major thrombin receptor PAR-1, because this receptor is expressed on numerous cell types within intra- and extravascular compartments of the injured lung.^{16,11} Current evidence supports a role for PAR-1 in promoting inflammation via the production of a host of proinflammatory mediators.^{17,18} We and others^{9,19,20,21} have shown that activation of PAR-1 by coagulation proteinases also promotes fibroblast proliferation, procollagen production, profibrotic mediator release, and differentiation into activated myofibroblasts *in vitro*, but to date, there have been no studies assessing the functional importance of this receptor in a lung injury model *in vivo*. In this report, we show for the first time that acute inflammatory and late fibrotic responses are dramatically attenuated in PAR-1-deficient mice in response to bleomycin injury, compared with correspondingly injured wild-type (WT) mice. We further show that these attenuated responses are accompanied by a reduction in the pulmonary levels of the key PAR-1-inducible proinflammatory and profibrotic mediators, monocyte chemoattractant protein-1 (MCP-1), connective tissue growth factor/fibroblast-inducible secreted protein-12 (CTGF/FISP12), and transforming growth factor- β -1 (TGF- β -1). Taken together, these data confirm a key role for PAR-1 in promoting inflammation and fibrosis in the injured lung and further highlight the contribution of coagulation proteinase cell signaling within intra- and extravascular compartments.

Materials and Methods

Materials

The generation and backcrossing of PAR-1^{-/-} mice, for 10 generations (>97%), onto the C57BL/6 background has

been described previously.^{22,23} The primers specific for mouse CTGF (FISP12) (NM010217), TGF- β -1 (NM011577), and the internal control 36B4 (BC011106) were designed using Beacon Designer Version 2.0 (Bio-Rad, Hercules, CA). The primer sequences for CTGF/FISP12 are: forward (548–565), 5'-GCAGCGGTGAGTCCTTCC-3'; reverse (779–758), 5'-AATGTGTCTTCCAGTCGGTAGG-3'. The primer sequences for TGF- β -1 are: forward (1707–1731), 5'-GGATACCAACTATTGCTTCAGCTCC-3'; reverse (1862–1838), 5'-AGGCTCCAAATATAGGGCAGGGTC-3'. The primer sequences for 36B4 are: forward (69–88), 5'-CGACCTGGAAGTCCAACTAC-3'; reverse (177–160), 5'-ATCTGCTGCATCTGCTTG-3'. The enzyme-linked immunosorbent assay (ELISA) for mouse interleukin (IL)-6 was from RnD Systems (Oxon, UK), and ELISA for mouse MCP-1 was from BD Biosciences, Pharmingen (San Diego, CA). The lactate dehydrogenase (LDH) activity assay kit was from Sigma-Aldrich (Poole, Dorset, UK). Antibodies specific for CTGF/FISP12 and TGF- β -1 (which recognizes latent and active TGF- β -1) were obtained from Santa Cruz Biotechnology, Inc. (San Diego, CA). Human-specific PAR-1 antibodies, raised against the peptide SFLLRNPNDKYEPF-NH₂ (abbreviated SFL) in rabbits and purified by affinity chromatography,²⁴ were a generous gift from Professor Eleanor Mackie (University of Melbourne, Melbourne, Australia). Human lung material was obtained from patients with fibrotic lung disease undergoing a diagnostic thoracoscopic biopsy or from patients undergoing a lobectomy for a solitary primary carcinoma. In lobectomies, normal lung tissue samples were taken from areas that were distant from tumor and that were histologically normal. The human studies were approved by the University College London Research Ethics Committee.

Animal Model of Pulmonary Fibrosis

Mice were housed in a specific pathogen-free facility, and all procedures were performed on mice between 6 and 8 weeks of age, in accordance with the UK Home Office Animals Scientific Procedures Act. Bleomycin (Lundbeck, Caldecotte, Milton Keynes, UK) or saline was administered by a single intratracheal injection (1 mg/kg body weight in 45 μ l of saline) under anesthesia.

For real-time reverse transcription polymerase chain reaction (RT-PCR) and lung inflammatory mediator analysis, lungs were carefully removed and immediately snap-frozen in liquid nitrogen. For assessment of total lung collagen and histological and immunohistochemical analyses, lungs were perfused with 3 ml of normal saline via the inferior vena cava. For measurement of total lung collagen, lungs were removed, blotted dry and the trachea and major airways were excised. Lungs were then weighed before being snap-frozen in liquid nitrogen. Lungs for histological and immunohistochemical analyses were fixed after perfusion: The trachea was cannulated, and lungs were insufflated with 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 25 cm H₂O, followed by removal of the heart and inflated lungs *en bloc* and immersion for 4 hours in fresh fixative. Subsequently, lungs were transferred to 15% sucrose in

PBS and left overnight at 4°C, before transfer to 70% ethanol. For BALF analysis, the trachea was cannulated via a ventral neck incision. Normal saline was instilled in 0.5-ml aliquots over 15 seconds, left *in situ* for 30 seconds, withdrawn over 15 seconds, and stored in polypropylene tubes on ice. The procedure was repeated five times, and >90% of the total instillate was recovered.

Determination of Total Lung Collagen

Total lung collagen was determined by measuring hydroxyproline content in aliquots of pulverized lung as described previously.^{25,26,27} Hydroxyproline was quantitated by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxao-1,3-diazole-derived acid hydrolysates. The secondary amino acid hydroxyproline reacts with 7-chloro-4-nitrobenzo-oxao-1,3-diazole to generate a chromophore with maximum light absorbance at 495 nm. Total lung collagen was calculated assuming that lung collagen contains 12.2% (w/w) hydroxyproline²⁸ and was expressed as milligrams of collagen per lung.

Histological Analysis

Individual lobes of mouse lungs or human biopsy material were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Before immunostaining, 5- μ m-thick lung tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS.

Masson's Trichrome Staining

Sections were stained for 10 seconds in celestine blue solution (0.5% celestine blue and 5% (w/v) ferric ammonium sulfate, both in water; and 14% (v/v) glycerin) (all from Sigma-Aldrich) and immersed for 10 seconds in Mayers hematoxylin, followed by 6 minutes in 1% (w/v) Ponceau red in water (both from BDH/Merck, Poole, Dorset, UK). Sections were differentiated in 1% (w/v) phosphomolybdic acid in water, before counterstaining with 0.5% (v/w) soluble methyl blue in 2.5% (v/v) acetic acid in water (both from Sigma-Aldrich). After staining, sections were dehydrated through increasing concentrations of ethanol and xylene before they were mounted in DPX (BDH/Merck). To demonstrate fibrin fibers, a modified staining technique, based on that of Lendrum et al²⁹ and involving a Ponceaux de Xylidine and Acid Fuchsin mix, was used.

Immunohistochemistry for CTGF/FISP12, TGF- β_1 , and PAR-1

Antigens were unmasked by microwaving sections in 10 mmol/L citrate buffer, pH 6.0 (twice for 10 minutes), and immunostaining was undertaken using the avidin-biotinylated enzyme complex method (Vector Laboratories, Burlingame, CA) with antibodies against CTGF (which also recognizes FISP12) at a concentration of 0.4 μ g/ml, TGF- β_1 at a concentration of 1 μ g/ml, SFL

(PAR-1) at a concentration of 0.54 μ g/ml, and equivalent concentrations of polyclonal nonimmune IgG controls. After incubation with the appropriate biotin-conjugated secondary antibody and subsequently with streptavidin solution, color development was performed using 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories) as a chromogen. Sections were counterstained using Gill-2 hematoxylin (Thermo-Shandon, Pittsburgh, PA), dehydrated, and mounted as described previously. Specificity of the signal obtained was confirmed by showing that no positive staining was detectable when the primary antibody was substituted with an equivalent concentration of nonimmune polyclonal IgG or when it was omitted altogether. In contrast to TGF β_1 , there are, to the best of our knowledge, no previous published reports of CTGF immunostaining in the bleomycin model. We therefore validated the monospecificity of the antibody used by confirming the presence of a single 38-kd immunoreactive band by Western-blot analysis of lung homogenates (data not shown).

Histological Scoring and Semiquantitative Immunohistochemical Image Analysis

Histological changes in lung tissue sections were assessed using the Ashcroft scoring system.³⁰ Two independent observers, blinded to the treatment group and genotype, examined all five lung lobes using light microscopy (400 $\times$ magnification) to determine a fibrosis score for each lobe. The mean Ashcroft fibrosis score was calculated as the average of the individual lobe fibrosis scores. Lung histology was also examined using semiquantitative image analysis, to evaluate the percentage of new collagen per lobe using a digital camera (JVC KY-F55BE; JVC, Yokohama, Japan) and KS300 software (Carl Zeiss Vision GmbH, Eching, Germany). The color intensity representative of new lung collagen after saline or bleomycin instillation was established after inspection of all sections, and a threshold was defined. Individual lobes were examined blind to experimental group (200 $\times$ magnification) using a BX40 microscope (Olympus, London, UK). For each field of view, the percentage of area staining of greater intensity than the defined threshold was taken to represent the percentage of new collagen. Fields were examined to cover the entirety of each lobe and were discarded if nonrepresentative areas such as airway lumen occupied >50% of the field of view. Between 2 (cardiac lobe) and 35 (left lobe) fields of view were analyzed for each lobe, depending on size. The data are expressed as mean percentage of new collagen per lobe. CTGF/FISP12 and TGF- β_1 immunostaining was also quantitated by image analysis (100 $\times$ magnification), and the data are expressed as mean percentage of positive immunostaining per lobe.

Assessment of BALF Total Cell Number, Differential Cell Counts, Total Protein, and LDH Activity

BALF cells were pelleted by centrifugation (300 $\times$ g for 10 minutes at 4°C). Pellets were resuspended in 1 ml of

Dulbecco's modified Eagle's medium (Gibco-BRL, Paisley, UK), and cells were counted with a standard hemocytometer. Differential cell counts were obtained of Diff-quick-stained (Dade Behring, Dudungen, Switzerland) cytospin preparations of the resuspended cells. Total protein and LDH activity in BALF supernatants were assessed per the manufacturers' protocols, using the bicinchoninic acid protein assay (Pierce, Rockford, IL) and an LDH activity assay kit (Sigma-Aldrich).

Lung Homogenate Analysis for IL-6 and MCP-1

Lung tissue homogenates were prepared based on a method described by Keane et al.^{31,32} Briefly, frozen lung powder was mixed with 0.5 ml of PBS with added protease inhibitors (Complete Mini; Roche Diagnostics, Sussex, UK) in polypropylene tubes. Samples were homogenized (three times for 20 seconds on ice) and filtered through a 1.2- μ m filter (Sartorius, Goettingen, Germany), and aliquots were frozen at -80°C until use. IL-6 and MCP-1 in homogenates were measured by ELISA (IL-6 sensitivity, 1.6 pg/ml; MCP-1 sensitivity, 15.6 pg/ml) according to manufacturers' instructions.

Real-Time RT-PCR Analysis of Lung Tissue for FISP12 and TGF- β_1

Total RNA was isolated from frozen powdered lung tissue with TRIzol reagent per the manufacturer's protocol (Gibco-BRL). Reverse transcription (RT) was carried out with 2 μ g of total RNA in a reaction volume of 20 μ l using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) following the provided instructions. Oligo (dT)₂₀ was used as the primer for RT. Real-time PCR was conducted using iQ SYBR Green Supermix on an iCycler iQ Real-time Detection System following the manual and analyzed using iCycler iQ Real-time PCR Detection System Software Version 3.0A (all from Bio-Rad). Cycling conditions were optimized such that the PCR efficiency was 100% ($\pm 5\%$). For CTGF/FISP12, each cycle was 95°C , 30 seconds; then 58°C , 30 seconds. For TGF- β_1 , each cycle was 95°C , 15 seconds; then 64°C , 45 seconds. For 36B4, each cycle was 95°C , 20 seconds; then 57.5°C , 30 seconds. The specificity of the PCR product was confirmed by melting curve analysis and gel electrophoresis. Single products of the expected size were obtained: 36B4, 109 bp; CTGF, 232 bp; TGF- β_1 , 155 bp. Cycle threshold (Ct) values were calculated from the amplification plots. CTGF/FISP12 mRNA levels in each sample were normalized for 36B4 using the equation $\text{FISP12}/36\text{B4} = 2^{\text{Ct}(36\text{B4}) - \text{Ct}(\text{FISP12})}$, allowing direct comparison between samples. Similarly, an equivalent equation was used to derive TGF- β_1 mRNA levels. The lowest mean value was then assigned an arbitrary value of 1, and the values of the other samples were scaled accordingly.

Statistical Analysis

All data in figures are presented as mean $\pm$ SEM, unless otherwise indicated. Statistical evaluation was performed

between individual treatment groups by one way analysis of variance with Student-Newman-Keuls method or Dunn's method for posthoc pair-wise multiple comparisons, using Sigmastat software. A *P* value of less than 0.05 was considered significant.

Results

Effect of PAR-1 Deficiency on BALF Inflammatory Cell Number and Vascular Leak in Response to Bleomycin Injury

The early response to bleomycin injury is characterized by a dramatic increase in microvascular leak and inflammatory cell recruitment.³³ The effect of PAR-1 deficiency on these processes was examined by assessing BALF total cell number and protein 6 days after bleomycin injury. BALF protein content was taken to represent vascular permeability and epithelial/endothelial barrier integrity.

BALF total cell numbers were similar in both genotypes given intratracheal saline. In contrast, the characteristic increase in BALF cell number induced by bleomycin in WT mice was attenuated by $56 \pm 10\%$ ($P < 0.05$) in PAR-1^{-/-} mice. (Figure 1A), although the change in relative proportions of inflammatory cells (lymphocytes, macrophages, and neutrophils) was similar in both mouse genotypes after bleomycin injury (data not shown).

There was no difference between BALF total protein levels in WT and PAR-1^{-/-} mice given intratracheal saline. The characteristic increase in BALF total protein induced by bleomycin in WT mice was attenuated by $53 \pm 12\%$ ($P < 0.05$) in PAR-1^{-/-} mice. (Figure 1B). These differences were accompanied by marked differences in lung weights, with the characteristic increase in lung weight induced by bleomycin in WT mice attenuated by $43.7 \pm 4.2\%$ ($P < 0.05$) in PAR-1^{-/-} mice (data not shown).

Attenuated responses in PAR-1^{-/-} mice are unlikely to be due to a difference in the immediate injury caused by bleomycin between the two mouse genotypes, because BALF LDH activity at 24 hours was increased to a similar extent in WT and PAR-1^{-/-} mice (Figure 1C).

Effect of PAR-1 Deficiency on Bleomycin-Induced Lung Fibrosis

The effect of PAR-1 deficiency on lung collagen accumulation, as an index of lung fibrosis, 14 days after intratracheal instillation of bleomycin or saline is shown in Figure 2. PAR-1^{-/-} and WT mice demonstrated no significant difference in lung collagen levels after saline challenge. However, lung collagen accumulation was reduced by $58.6 \pm 5.5\%$ in bleomycin-instilled PAR-1^{-/-} mice compared with WT mice given bleomycin ($P < 0.05$) (Figure 2G).

Lung histopathological changes after bleomycin instillation were also assessed at this time point. For saline-instilled mice, lung histological appearance was entirely normal (Figure 2, A and B). In contrast, lung tissue sections from WT mice given bleomycin showed extensive

Figure 1. Increases in BALF total inflammatory cell number and total protein concentration in response to bleomycin are attenuated in PAR-1^{-/-} mice. **A:** Total number of inflammatory cells in BALF obtained 6 days after intratracheal instillation of saline or bleomycin. **B:** Total protein concentration in BALF obtained 6 days after intratracheal instillation of saline or bleomycin. **C:** LDH activity in BALF obtained 24 hours after intratracheal instillation of saline or bleomycin. Data represent the mean ± SEM of values obtained in five (C) or eight (A and B) mice per group. *, $P < 0.05$ difference between bleomycin-treated PAR-1^{-/-} and bleomycin-treated WT mice. †, $P < 0.05$ comparison with saline-treated mice.

patchy areas of regional interstitial fibrosis with marked disruption of the alveolar unit and increased deposition of collagen (Figure 2C). In PAR-1^{-/-} mice, the structural integrity of the lung was less severely affected after bleo-

mycin injury, with less evidence of fibrotic obliteration and destruction of alveolar units (Figure 2D).

The severity of fibrosis was also assessed by Ashcroft scoring and semiquantitative image analysis (evaluation of the percentage of new collagen per lobe), using previously described methods.²⁷ These assessments confirmed that the severity of fibrosis and the percentage of newly synthesized collagen content per lobe were significantly reduced in bleomycin-instilled PAR-1^{-/-} mice compared with correspondingly injured WT mice ($P < 0.05$) (Figure 2, E and F).

Effect of PAR-1 Deficiency on Lung MCP-1 and IL-6 in Response to Bleomycin Injury

To begin to examine the mechanisms by which PAR-1 deficiency affords protection from acute inflammation in this model, we assessed pulmonary levels of two potent PAR-1-inducible mediators known to be elevated after bleomycin administration. Lung homogenates were prepared from all treatment groups at 6 days, and MCP-1 and IL-6 levels were determined by ELISA.

There was no significant difference in lung MCP-1 levels after saline treatment between the two mouse genotypes (Figure 3A). MCP-1 levels were increased in both genotypes in response to bleomycin injury, but this increase was attenuated by $35.1 \pm 13.6\%$ for bleomycin-treated PAR-1^{-/-} mice compared with WT mice given bleomycin ($P < 0.05$). In contrast, there were similar increases in IL-6 levels in bleomycin-treated WT and PAR-1^{-/-} mice compared with saline-treated controls (Figure 3B).

Effect of PAR-1 Deficiency on CTGF/FISP12 and TGF- β_1 Expression in Response to Bleomycin Injury

We next assessed pulmonary levels of two potent PAR-1-inducible profibrotic mediators known to be elevated in this model. Figures 4 and 5 show representative immunohistochemical staining for CTGF/FISP12 and TGF- β_1 after intratracheal instillation of bleomycin or saline in WT and PAR-1^{-/-} mice at 14 days. CTGF/FISP12 immunostaining was predominantly localized to bronchiolar epithelium in saline control mice (Figure 4, A and B). In bleomycin-instilled mice, immunostaining was associated with infiltrating macrophages and interstitial spindle-shaped cells (Figure 4, C and D) but appeared less intense in PAR-1^{-/-} than in WT mice.

In contrast, TGF- β_1 immunostaining was predominantly localized to alveolar macrophages and to both bronchiolar and alveolar epithelium in saline control mice (Figure 5, A and B). In bleomycin-instilled mice, the intensity of staining increased markedly and was associated with the alveolar wall, infiltrating macrophages, and extracellular matrix associated with fibrotic foci (Figure 5, C and D) but appeared less intense in PAR-1^{-/-} than WT mice.

Figure 3. PAR-1^{-/-} mice display reduced lung levels of MCP-1 but not IL-6 following intratracheal bleomycin. Figure shows levels of MCP-1 (**A**) and IL-6 (**B**), as measured by ELISA, in lung homogenates 6 days after intratracheal saline or bleomycin. Data represent the mean $\pm$ SEM of values obtained in eight mice per group. *, $P < 0.05$, difference between bleomycin-treated PAR-1^{-/-} and bleomycin-treated WT mice. †, $P < 0.05$ comparison with saline-treated mice.

Semiquantitative image analysis revealed that CTGF/FISP12 and TGF- β_1 immunoreactivity was increased after bleomycin injury in both mouse genotypes ($P < 0.05$) (Figures 4E and 5E) and further that following bleomycin, PAR-1 deficiency was associated with $63.1 \pm 4.2\%$ and $60.4 \pm 6.0\%$ reductions in CTGF/FISP12 and TGF- β_1 immunoreactivity ($P < 0.05$) relative to correspondingly instilled WT mice. Of interest, there was also less CTGF/FISP12 and TGF- β_1 immunoreactivity ($P < 0.05$) in saline-instilled PAR-1^{-/-} mice compared with saline-instilled WT mice.

We also assessed CTGF/FISP12 and TGF- β_1 mRNA levels in bleomycin-treated PAR-1^{-/-} and WT mice at 6 and 14 days using quantitative real-time RT-PCR. PAR-1 deficiency had no effect on CTGF/FISP12 mRNA levels at baseline (saline treatment). At 6 and 14 days, CTGF/

Figure 2. Lung collagen accumulation in response to bleomycin is attenuated in PAR-1^{-/-} mice. **A to D:** Representative lung tissue sections from PAR-1^{-/-} and WT mice 14 days after saline and bleomycin instillations (black and white reproductions; original magnification, $\times 40$). **A and B:** Lung architecture was normal in both mouse genotypes given saline. **C:** Extensive patchy fibrotic foci with increased deposition of collagen were seen in WT mice given bleomycin. **D:** The severity of fibrosis appeared much reduced in bleomycin-treated PAR-1^{-/-} relative to bleomycin-treated WT mice. **E and F:** Ashcroft scoring and semiquantitative image analysis demonstrating severity of fibrosis and percentage of newly synthesized collagen content per lobe in response to bleomycin. **G:** Total lung collagen, as measured by reverse phase HPLC quantitation of lung hydroxyproline in acid hydrolysates of pulverized lung. **E to G:** Data represent the mean $\pm$ SEM of values obtained in groups of six (**E** and **F**) or eight mice (**G**). *, $P < 0.05$ difference between bleomycin-treated PAR-1^{-/-} and bleomycin-treated WT mice. †, $P < 0.05$ comparison with saline-treated mice.

Figure 4. Immunostaining for FISP12 is reduced in the lungs of PAR-1^{-/-} mice. **A to D:** Immunostaining for FISP12 on representative lung tissue sections from PAR-1^{-/-} and WT mice 14 days after saline and bleomycin instillations; original magnification, ×200. **A and B:** In saline-instilled mice, positive immunostaining (brown) was predominantly localized to bronchiolar epithelium. **C and D:** In bleomycin-instilled mice, immunostaining was also associated with alveolar macrophages, interstitial spindle-shaped cells, and adjacent extracellular matrix but appeared less intense in PAR-1^{-/-} mice. **E:** Quantitation of immunostaining (semiquantitative immunohistochemical image analysis). Data represent mean ± SEM of values obtained in at least four mice per group. *, $P < 0.05$ difference between PAR-1^{-/-} and WT mice after instillation of saline or bleomycin. †, $P < 0.05$ difference in bleomycin-instilled compared with saline-instilled mice of the same genotype.

FISP12 mRNA levels were increased by 3.6 ± 1.1 -fold and 3.4 ± 0.7 -fold ($P < 0.05$) in bleomycin-instilled WT mice relative to saline-instilled controls. At 6 days following bleomycin, PAR-1 deficiency was associated with a reduction of $78.8 \pm 5.0\%$ ($P < 0.05$) in CTGF/FISP12 mRNA levels relative to correspondingly injured WT mice. At 14 days, there was a trend for a reduction in CTGF/FISP12 mRNA levels of $43.7 \pm 11.4\%$ in PAR-1^{-/-} mice (Figure 6, A and B), but this failed to reach statistical significance. TGF- β_1 mRNA levels were similar in the two genotypes at baseline, and there was a nonsignificant trend toward an increase in response to bleomycin in WT mice but not in PAR-1^{-/-} mice (data not shown). Finally, we also assessed fibrin deposition in the lung at a time point (7 days) at which thrombin levels are maximally elevated in this model.¹⁰ There was no difference in the extent of fibrin deposited within inflammatory and fibrotic

Figure 5. Immunostaining for TGF- β_1 is reduced in the lungs of PAR-1^{-/-} mice. **A to D:** Immunostaining for TGF- β_1 on representative lung tissue sections from PAR-1^{-/-} and WT mice 14 days after saline and bleomycin instillations; original magnification, ×200. **A and B:** In saline-treated mice, positive immunostaining (brown) was localized to bronchiolar epithelium, alveolar macrophages, and alveolar epithelium. **C and D:** In bleomycin-treated mice immunostaining increased markedly on the alveolar wall, infiltrating macrophages, and extracellular matrix associated with fibrotic foci but appeared less intense in PAR-1^{-/-} mice. **E:** Quantitation of immunostaining (semiquantitative immunohistochemical image analysis). Data represent mean ± SEM of values obtained in at least four mice per group. *, $P < 0.05$ difference between PAR-1^{-/-} and WT mice after instillation of saline or bleomycin. †, $P < 0.05$ difference in bleomycin-instilled compared with saline-instilled mice of the same genotype.

foci between bleomycin-instilled PAR-1^{-/-} and WT mice (data not shown).

Immunohistochemical Expression of PAR-1 in Human Lung Biopsy Specimens

The data obtained in the bleomycin model support the hypothesis that PAR-1 plays an important role in driving the inflammatory and fibrotic responses to bleomycin injury. To begin to examine the potential role of this receptor in fibrotic lung diseases, we examined the expression of PAR-1 in a limited number of control and fibrotic human lung biopsy specimens ($n = 3$) by immunohistochemistry (Figure 7). In control lung tissue, there was weak staining for PAR-1 associated with resident alveolar macro-

Figure 6. Pulmonary FISP12 mRNA levels induced in response to bleomycin are attenuated in PAR-1^{-/-} mice. Relative abundance of CTGF/FISP12 mRNA, measured by real-time RT-PCR, is normalized for internal control gene 36B4 and expressed in arbitrary units. **A:** Relative abundance of CTGF/FISP12 mRNA in the lung 6 days after intratracheal saline or bleomycin. **B:** Relative abundance of CTGF/FISP12 mRNA in the lung 14 days after intratracheal saline or bleomycin. Data represent the mean $\pm$ SEM of values obtained in eight mice per group. *, $P < 0.05$, difference between bleomycin-treated PAR-1^{-/-} and bleomycin-treated WT mice. [†], $P < 0.05$ comparison with saline-treated mice.

phages (Figure 7A). In contrast, in lung tissue obtained from patients with established pulmonary fibrosis (Figure 7B), there was intense and widespread staining associated with macrophages and also with elongated spindle-shaped cells with a typical fibroblast morphology within fibroproliferative and inflammatory foci. Similar strong PAR-1 immunostaining was observed for all three fibrotic lung biopsy specimens examined. Figure 7C shows that there was no detectable signal for a similar section stained with a nonimmune polyclonal rabbit IgG control.

Discussion

Activation of the coagulation cascade is commonly observed in the lungs of patients with acute and chronic inflammatory and fibrotic lung disorders.³⁴ PAR-1 exerts potent proinflammatory and profibrotic effects *in vitro*, after activation by coagulation proteinases such as thrombin and factor Xa. However, the role of this receptor in the response to lung injury *in vivo* is currently unknown. This study provides evidence that PAR-1 deficiency is associated with a dramatic reduction in the acute inflammatory and the subsequent chronic fibrotic response to lung injury induced by intratracheal instillation of bleomycin.

Figure 7. Immunohistochemical localization of PAR-1 in human biopsy specimens. Immunostaining for PAR-1 on representative lung tissue sections from human biopsy material; original magnification, $\times 400$. **A:** Weak immunostaining for PAR-1 was associated with resident alveolar macrophages (arrow M) in normal lung. **B:** Intense immunostaining for PAR-1 in fibrotic lung was associated with macrophages (arrow M) in fibroproliferative and inflammatory foci and also with elongated spindle-shaped cells with a typical fibroblast morphology (arrow F). **C:** Use of polyclonal nonimmune IgG control instead of SFL (PAR-1) antibody demonstrated no immunostaining of a section of lung from a patient with pulmonary fibrosis.

PAR-1^{-/-} Mice Are Protected from Bleomycin-Induced Lung Inflammation

Bleomycin-induced lung injury is associated with recruitment of inflammatory cells into the injured lung during the first 7 days.³³ This is accompanied by a rapid increase in pulmonary microvascular leak, which is manifest as an increase in total protein in bronchoalveolar lavage fluid.³⁵ These responses are temporally correlated with maximal levels of intra-alveolar coagulation proteinase activity (thrombin levels)^{10,36} and expression of PAR-1 on inflammatory cells in the bleomycin-injured lung.¹¹

Assessment of these early inflammatory responses in the present study revealed that increases in BALF total inflammatory cell number and total protein levels were attenuated by 56% and 53% respectively in bleomycin-instilled PAR-1^{-/-} mice compared with correspondingly injured WT mice. Assessment of LDH activity and the extent of fibrin deposited within the lung revealed similar increases for both genotypes, suggesting that these attenuated responses were not due to any difference in direct injury caused by bleomycin or in fibrin formation between the two mouse genotypes.

There are a number of potential mechanisms by which PAR-1 may contribute to the inflammatory responses in this model. Activation of this receptor by either thrombin or immediate upstream coagulation proteinases on a variety of cell types within intra- and extravascular compartments can induce the production and release of a host of proinflammatory mediators, including among others MCP-1, IL-1 β , IL-6, and IL-8.^{37,38,39,18} Activation of PAR-1 by thrombin may also influence inflammatory cell trafficking in this model *via* its ability to induce the expression of endothelial cell surface adhesion molecules, such as E- and P-selectin and intercellular adhesion molecule-1 (ICAM-1).^{40,41} In terms of influencing BALF protein levels, thrombin and PAR-1 peptide agonists have been shown to increase vascular permeability by direct and nitric oxide-dependent effects on endothelial cells⁴² and *via* the release of histamine from mast cells.⁴³ The necessity for PAR-1 in mediating these responses was further confirmed in experiments in which thrombin-induced increases in pulmonary microvascular permeability were shown to be abrogated in isolated lung preparations obtained from PAR-1^{-/-} mice.⁴⁴ It is noteworthy that in humans, PAR-1 activation of platelets also increases vascular permeability *via* the release of vasoactive mediators; however, platelet-derived mediators are unlikely to be influenced by PAR-1 deficiency in the present study because murine platelet responses are mediated *via* a dual PAR-3/PAR-4 receptor system.⁴⁵

To begin to delineate the potential mechanism by which PAR-1^{-/-} mice were protected from bleomycin-induced lung inflammation, we measured the pulmonary levels of two PAR-1-inducible mediators (MCP-1 and IL-6) that have previously been implicated in the inflammatory response in this model of lung injury.^{46,47} IL-6 levels were found to be increased to the same extent in response to bleomycin injury in both mouse genotypes, suggesting that the up-regulation of this cytokine occurs

via a PAR-1-independent pathway in this model and furthermore that the protective response observed in PAR-1^{-/-} mice cannot be explained by an attenuated IL-6 response. Our findings for IL-6 levels are further in agreement with a recent study in a mouse model of sepsis, in which deficiency of PAR-1 or PAR-2 alone had the same effect on IL-6 expression after endotoxic insult as observed for WT mice. However, when PAR-2 deficiency was combined with hirudin, thus additionally blocking signaling through PAR-1 and PAR-4, IL-6 levels were reduced, suggesting that multiple PARs may be responsible for the regulation of this proinflammatory mediator in this disease model.⁴⁸ In contrast to IL-6, MCP-1 levels were significantly reduced in bleomycin-instilled PAR-1^{-/-} mice compared with correspondingly injured WT mice in our study. In terms of the functional significance of this observation, anti-MCP-1 antibodies have been shown to reduce leukocyte accumulation after bleomycin-induced lung injury,⁴⁶ suggesting that this chemokine plays an important role in the inflammatory response to bleomycin. Although multiple PARs may mediate the cross-talk between coagulation and inflammation in certain disease settings, our findings suggest that PAR-1 plays a critical role in this cross-talk in this injury model.

PAR-1^{-/-} Mice Are Protected from Bleomycin-Induced Lung Fibrosis

Assessment of the late fibrotic response to bleomycin injury revealed that total lung collagen accumulation was attenuated by about 60% in PAR-1^{-/-} mice instilled with bleomycin compared with correspondingly injured WT mice. The large focal areas of interstitial fibrosis observed histologically in WT mice after bleomycin instillation were significantly reduced in PAR-1^{-/-} mice, as determined by Ashcroft scoring and by semiquantitative image analysis.

The observed attenuation in bleomycin-induced MCP-1 levels in PAR-1^{-/-} mice in our study may also be relevant to the reduced lung collagen accumulation in these mice. In addition to influencing inflammatory cell recruitment, there is evidence that MCP-1 may act as a profibrotic mediator by promoting fibroblast procollagen gene expression *via* up-regulation of TGF- β .⁴⁹ More recently, mice lacking the CC chemokine receptor CCR2 (the major MCP-1 receptor) have been shown to be protected from bleomycin and fluorescein isothiocyanate-induced pulmonary fibrosis.^{50,51} Of note, the reduction in lung collagen accumulation observed in CCR2-deficient mice appeared to occur without significant attenuation of the preceding inflammatory response.⁵¹ Moreover, anti-MCP-1 gene therapy administered at early and late phases of the bleomycin response suggest that this chemokine plays an important role during the chronic fibrotic phase.⁵²

We also examined whether the expression of two other PAR-1-inducible profibrotic mediators, TGF- β_1 and CTGF/FISP12, was attenuated in PAR-1^{-/-} mice. TGF- β_1 is widely accepted as one of the key profibrotic mediators in this model⁵³ and in patients with fibrotic lung dis-

ease.^{54,55} It is one of the most potent inducers of fibroblast procollagen production examined to date and promotes the differentiation of fibroblasts into highly activated myofibroblasts, the predominant fibroblast phenotype present in active fibrotic lesions.⁵⁶ Although the evidence supporting a role for CTGF/FISP12 is currently not as strong as for TGF- β_1 , this mediator is also highly expressed in this model^{57,11} and in patients with fibrotic lung disease.^{58,59} CTGF/FISP12 also exerts profibrotic effects *in vitro* by directly stimulating fibroblast function, and overexpression of CTGF by adenoviral gene transfer has been shown to induce transient pulmonary fibrosis in mice.⁶⁰ There is also evidence suggesting that CTGF may act synergistically with TGF- β to promote long-term tissue fibrosis.⁶¹

In the present study, TGF- β_1 and CTGF/FISP12 immunoreactivity were significantly reduced in bleomycin-instilled PAR-1^{-/-} mice. This immunoreactivity reflects both positive staining from resident lung cells, as well as by locally activated cells, and recruited inflammatory cells. However, a difference in immunoreactivity between lungs of bleomycin-treated WT and PAR-1^{-/-} mice was apparent for the interstitium and the alveolar wall, independently of whether there was evidence of increased cell number or matrix accumulation. Although this suggests that there may be a difference in local up-regulation of CTGF and TGF- β_1 between the two mouse genotypes, further studies (eg, *in situ* hybridization) would be required to confirm this observation.

The changes in immunoreactivity were also preceded by a significant reduction in CTGF/FISP12 mRNA levels on day 6. This observation is in agreement with our previous report that direct thrombin inhibition attenuates bleomycin-induced lung collagen accumulation and CTGF mRNA levels¹¹ and further suggests an important role for thrombin as a major PAR-1 activator in this model. In contrast, although TGF β_1 protein expression in the injured lung was reduced by more than one-half in bleomycin-instilled PAR-1^{-/-} mice compared with correspondingly injured WT mice at day 14, we were unable to detect a change in TGF β_1 mRNA expression at 6 or 14 days (data not shown). Although not a universal finding, this lack of an effect at the mRNA level has previously been reported by others. For example, Kaminski et al⁶² were unable to detect increases in TGF- β_1 , - β_2 , and - β_3 mRNA in this model, even though the signature gene expression profile associated with increased TGF- β signaling was clearly evident. We believe that the observation that PAR-1 deficiency is associated with reduced TGF- β_1 protein levels in response to bleomycin injury points to the possibility that this may represent an important pathway by which PAR-1 activation contributes to the fibrotic phase in this model. Thrombin and/or PAR-1 agonists, have been shown to stimulate the release of TGF- β from a number of cell types, including vascular smooth muscle cells and kidney mesangial and epithelial cells,^{63–66} and we have recently confirmed this observation for lung epithelial cells (R.H. Johns, G.J. Laurent, and R.C. Chambers, unpublished data). The potential importance of PAR-1 signaling in the lung is further suggested by our observation that PAR-1 deficiency also appears to

be associated with reduced immunoreactivity for CTGF/FISP12 and TGF- β_1 in saline control mice. However, although there is good evidence that PAR-1 activation on a number of cell types influences the production of profibrotic mediators, we cannot rule out the possibility that protection from bleomycin-induced fibrosis in PAR-1^{-/-} mice results, at least in part, from an attenuated early inflammatory response.

To begin to examine the potential role of PAR-1 in human fibrotic lung disease, we examined PAR-1 expression in a limited number of biopsy specimens from patients with established pulmonary fibrosis. Our findings of strong immunostaining in pulmonary fibrosis biopsies are in accord with our previous findings in the bleomycin model¹¹ and support the hypothesis that PAR-1 signaling in response to coagulation proteinases within extravascular compartments may contribute to the development of fibrosis in patients.

There are a number of potential mechanisms by which PAR-1 expression may be elevated in the injured lung because expression of this receptor is known to be modulated by a variety of mediators and stimuli, including thrombin and TGF- β .^{67,68} Furthermore, monocyte differentiation has been shown to correlate with increased PAR-1 expression *in vitro*.⁶⁹ Importantly, activation of PAR-1 by thrombin has also been shown to induce fibroblast to myofibroblast differentiation,²¹ and PAR-1 expression by these cells has been shown to be increased *in vitro* and *in vivo*.⁷⁰ Myofibroblast differentiation is also influenced by TGF- β ⁷¹ and although not performed in this study, future experiments to investigate the role of this process in bleomycin-treated PAR-1^{-/-} mice may generate further interesting observations.

Conclusions and Clinical Implications of This Study

Activation of the coagulation cascade is a feature of a number of lung diseases associated with inflammation and excessive deposition of extracellular matrix proteins, including idiopathic pulmonary fibrosis,^{72–74} pulmonary fibrosis associated with systemic sclerosis,^{9,8} acute lung injury/acute respiratory distress syndrome,⁷⁵ chronic lung disease of prematurity,⁷⁶ cryptogenic organizing pneumonia,⁷⁷ and airway remodeling in asthma.⁷⁸ The findings reported in the present study are, to our knowledge, the first to demonstrate a central role for PAR-1 during the inflammatory and fibrotic phases of experimentally induced lung injury. Taken together with the previous observation that direct thrombin inhibition attenuates bleomycin-induced lung collagen accumulation¹¹ and the lack of protection of fibrinogen-null mice,¹³ our findings suggest a central role for thrombin-dependent PAR-1 signaling in this model. This report adds further evidence to existing studies suggesting an important function for this receptor in driving inflammation and abnormal remodeling in a number of disease settings, including restenosis and neointima formation after vascular injury,⁷⁹ renal inflammation and crescentic glomerulonephritis,²³ and liver fibrosis.⁸⁰ This evidence places PAR-1

as one of the critical receptors involved in orchestrating the interplay between coagulation, inflammation, and remodeling in response to tissue injury in intra- and extravascular compartments, in addition to its well-recognized role in thrombosis.⁸¹ Strategies aimed at blocking PAR-1 in the context of acute and chronic forms of inflammatory and fibrotic lung disease may represent an exciting new opportunity for the treatment of these conditions in the future.

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