

Increased expression of protease nexin-1 in fibroblasts during idiopathic pulmonary fibrosis regulates thrombin activity and fibronectin expression

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Idiopathic pulmonary fibrosis (IPF) is a chronic diffuse lung disease characterized by an accumulation of excess fibrous material in the lung. Protease nexin-1 (PN-1) is a tissue serpin produced by many cell types, including lung fibroblasts. PN-1 is capable of regulating proteases of both coagulation and fibrinolysis systems, by inhibiting, respectively, thrombin and plasminergic enzymes. PN-1 is thus a good candidate for regulating tissue remodeling occurring during IPF. We demonstrated a significant increase of PN-1 expression in lung tissue extracts, lung fibroblasts and bronchoalveolar lavage fluids of patients with IPF. The increase of PN-1 expression was reproduced after stimulation of control lung fibroblasts by transforming growth factor- β , a major pro-fibrotic cytokine involved in IPF. Another serpin, plasminogen activator inhibitor-1 (PAI-1) is also overexpressed in fibrotic fibroblasts. Unlike PAI-1, cell-bound PN-1 as well as secreted PN-1 from IPF and stimulated fibroblasts were shown to inhibit efficiently thrombin activity, indicating that both serpins should exhibit complementary roles in IPF pathogenesis, via their different preferential antiprotease activities. Moreover, we observed that overexpression of PN-1 induced by transfection of control fibroblasts led to increased fibronectin expression, whereas PN-1 silencing induced in fibrotic fibroblasts led to decreased fibronectin expression. Overexpression of PN-1 lacking either its antiprotease activity or its binding capacity to glycosaminoglycans had no effect on fibronectin expression. These novel findings suggest that modulation of PN-1 expression in lung fibroblasts may also have a role in the development of IPF by directly influencing the expression of extracellular matrix proteins. Our data provide new insights into the role of PN-1 in the poorly understood pathological processes involved in IPF and could therefore give rise to new therapeutic approaches.

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Idiopathic pulmonary fibrosis (IPF) is a chronic diffuse lung disease characterized by progressive deterioration in lung function ultimately leading to death. An important key event in IPF is excessive accumulation of extracellular matrix (ECM) components, resulting from an imbalance between matrix synthesis and degradation. Therefore the modulation of ECM proteolysis has a major regulatory role in the development of IPF and depends on the action of proteases, such as matrix metalloproteinases or proteases of the plasminergic system.

The control of protease activities is thus important in the homeostasis of the lung. Endogenous inhibitors, and in particular serine protease inhibitors (serpins), are known

to have such a central role. Serpins form a large family of structurally related proteins present in the plasma or in tissues. They are suicide inhibitors using a unique and extensive conformational change to inhibit proteases.¹ Among serpins, plasminogen activator inhibitor-1 (PAI-1)^{2,3} has been implicated in the pathophysiology of IPF. The level of plasminogen activation within the lung is indeed linked to the extent of fibrosis during IPF, as illustrated by the decrease in collagen accumulation observed after bleomycin-induced lung injury in mice with enhanced lung urokinase plasminogen activator (uPA) activity.⁴ We hypothesized that another serpin named protease nexin-1 (PN-1) or serpinE2, which is barely detectable in plasma but produced by most

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cell types, in particular human lung fibroblasts,⁵ might be a good candidate for the regulation of lung remodeling during IPF. Indeed, PN-1 is an inhibitor of uPA and tissue plasminogen activator but has a larger inhibition spectrum than PAI-1, as it is also able to efficiently inhibit thrombin and plasmin.^{6,7} Moreover, PN-1 is thought to have a pathogenic role in the development of scleroderma, another fibrotic disease.⁸ PN-1 is thus a good candidate for regulating both ECM and fibrin deposition in tissues.

Although PN-1 was shown to be expressed in the normal adult mouse lung⁹ and in the normal and pathological human lung,¹⁰ there have been no studies investigating the role of PN-1 in pulmonary fibrosis. Our goal was thus to examine the expression of PN-1 in lungs and in fibrotic human pulmonary fibroblasts from patients with IPF. Moreover, because transforming growth factor- β (TGF- β) is a key pro-fibrotic cytokine implicated in IPF, known to regulate ECM production, cell growth, and tissue repair,¹¹ we analyzed the regulation of PN-1 expression by TGF- β in pulmonary fibroblasts and the effect of such PN-1 modulation on the behavior of these cells.

MATERIALS AND METHODS

Ethics Statement

Human samples were used with the approval of the Institutional Review Board of the French learned society for respiratory medicine (Société de Pneumologie de Langue Française). Oral informed consent was obtained from the participants to the study at the time of bronchoscopy and recorded in the clinical charts. Clinical and functional data were collected on a standardized and anonymous collection form.

Human Samples

IPF was diagnosed according to the 2011 international statement. Bronchoalveolar lavages (BAL) were performed and lavage fluid processed as previously described.¹² Fibrotic lung biopsies were derived from patients with IPF, and normal lung samples were obtained from a non-involved lung segment from patients undergoing lung surgery for a primary lung tumor.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded sections of human lung tissue were used for immunohistochemical studies. The monoclonal anti-PN-1 IgG (1F6; 4 μ g/ml) previously described,¹³ an anti- α -smooth muscle actin IgG (Dako France SAS, Les Ulis, 4 μ g/ml), or an isotype-matched mouse IgG1 (Dako France SAS) antibody was used for immunohistochemistry. Positive staining was revealed using the Vectastain ABC-alkaline phosphatase kit mouse IgG (Vector Eurobio/Abcys, Courtaboeuf, France) and the fast red substrate (Dako France SAS).

Culture of Fibroblasts and TGF- β Stimulation

Control pulmonary fibroblasts were obtained from LGC standards human cell lines (LGC Standards SARL, Molsheim, France). Fibrotic human fibroblasts were isolated from lung tissue from patients with IPF and cultured (IPF fibroblasts) as previously described.¹⁴ Briefly, lung tissue samples were obtained by open lung biopsy. Fibroblasts were cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum supplemented with antibiotics. Human lung fibroblasts were cultured until passage six and were incubated in the presence or not of TGF- β (2–30 ng/ml, R&D Systems Europe, Lille, France) for 6–48 h.

Reverse Transcription and Quantitative Real-time PCR

Total RNA was isolated from fibroblasts using Trizol reagent (Invitrogen/Life Technologies SAS, Saint Aubin, France), according to the manufacturer's directions. Double-stranded cDNAs were synthesized and amplified as previously described.¹⁵ Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science, Meylan, France). The following run protocol for PN-1, PAI-1, collagen 1 α 2, collagen 3 α 1, and fibronectin was used: denaturation: 95 °C, 5 min; and amplification and quantitation (40 cycles): 60 °C, 10 min; 72 °C, 30 min. Fluorescence of the samples was monitored continuously while the temperature was increased from 62 to 95 °C at a linear transition rate of 0.1 °C/s, and arrested by a final cooling step at 45 °C. PN-1 primers were: forward 5'-GGGCAYCCTGGGCTAMACTG A-3' and reverse 5'-TGCTCTTGCTGGGGCTGGA-3'; PAI-1 primers were: forward 5'-GCACAACCCACAGGAAC-3' and reverse 5'-TGCTTCAAACCTCTCTCCCAG-3'; collagen 1 α 2 primers were: forward 5'-CGAGGTAGTCTTTTCAGCAA CACAGT-3' and reverse 5'-TTGAGACTCAGCCACCCAGA GT-3'; collagen 3 α 1 primers were: forward 5'-AGCTGGAAAG AGTGGTGACAG-3' and reverse 5'-CCTTGAGGACCAGGA GCAC-3'; and fibronectin primers were: forward 5'-CTGGC CGAAAATACATTGTAAA-3' and reverse 5'-CCACAGTCGG GTCAGGAG-3'. LightCycler run protocol for GAPDH was as follows: denaturation: 95 °C, 5 min; and amplification and quantitation (40 cycles): 65 °C, 10 min; 72 °C, 20 min. GAPDH primers were: forward 5'-GTGAAGGTCGGAGTCA CG-3' and reverse 5'-GGTGAAGACGCCAGTGGACTC-3'. Transcripts levels were normalized to GAPDH mRNA.

Immunoblot Analysis

Culture media and cell lysates were collected from TGF- β -stimulated and IPF fibroblasts. Proteins were extracted by cell lysis in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl; 3 mM EDTA, 0.1% SDS, 1% Nonidet P-40) containing a protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) and centrifuged at 5000 g for 5 min at 4 °C; samples were then analyzed by immunoblotting. Protein concentrations were measured using a BCA Pierce protein assay (Interchim, Montluçon, France). Proteins were solubilized with 2% SDS and separated by

electrophoresis on 10% acrylamide SDS gel. Immunoblots were performed using the monoclonal anti-PN-1 IgG (1F6; 2 µg/ml), a monoclonal anti-fibronectin IgG (1 µg/ml; Santa Cruz/CliniSciences, Nanterre, France), and a monoclonal anti-GAPDH (1 µg/ml; Abcam) followed by horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) at a 1:50000 dilution. Immunoreactivity was visualized by chemiluminescence (ECL; Amersham/VWR International, Fontenay-sous-bois, France).

Thrombin Assay

Confluent control fibroblasts were serum-starved overnight and incubated for 24 h with serum-free medium containing 10 ng/ml of TGF-β. Confluent IPF fibroblasts were only serum-starved overnight and incubated for 24 h with serum-free medium. At the end of the incubation, conditioned media (CM) were harvested for analysis. CM were incubated with 0.2 nM human α-thrombin (purified as previously described¹⁶) for 30 min at 37 °C in the presence or absence of a blocking polyclonal anti-PN-1 IgG (100 µg/ml)¹⁷ or a blocking monoclonal anti-PAI-1 IgG (100 µg/ml) (MA-33B8-307; Molecular Innovations/Gentaur Europe

BVBA, Kampenhout, Belgium). At the end of incubation, the thrombin chromogenic substrate S2238 (Cryo pep, Montpellier, France) was added at a final concentration of 0.2 mM, and changes in the absorbance were recorded at 405 nm. Residual thrombin activity is expressed as the ratio of the activity measured in the presence of the CM derived from controls, TGF-β-stimulated, or IPF fibroblasts to the activity measured in the presence of DMEM medium culture × 100. The experimental procedure realized as described above was also performed directly on cultured cells.

Plasmid and siRNA Transfection

The pcDNA3 vectors containing the coding sequence for the wild-type PN-1, the inactive mutant PN-1 (mutated active site), or the variant of PN-1 in which the heparin site is nonfunctional (mutated glycosaminoglycans (GAG) binding site) were produced as previously described.¹⁸ Control fibroblasts were transfected with an empty pcDNA3 vector (mock vector) or vectors containing the different PN-1 coding sequences by using the XtremGene kit (Roche Applied Science) according to the manufacturer's instructions. Fibroblasts were assayed after 48 h of transfection to quantify PN-1, collagen I, and fibronectin expression.

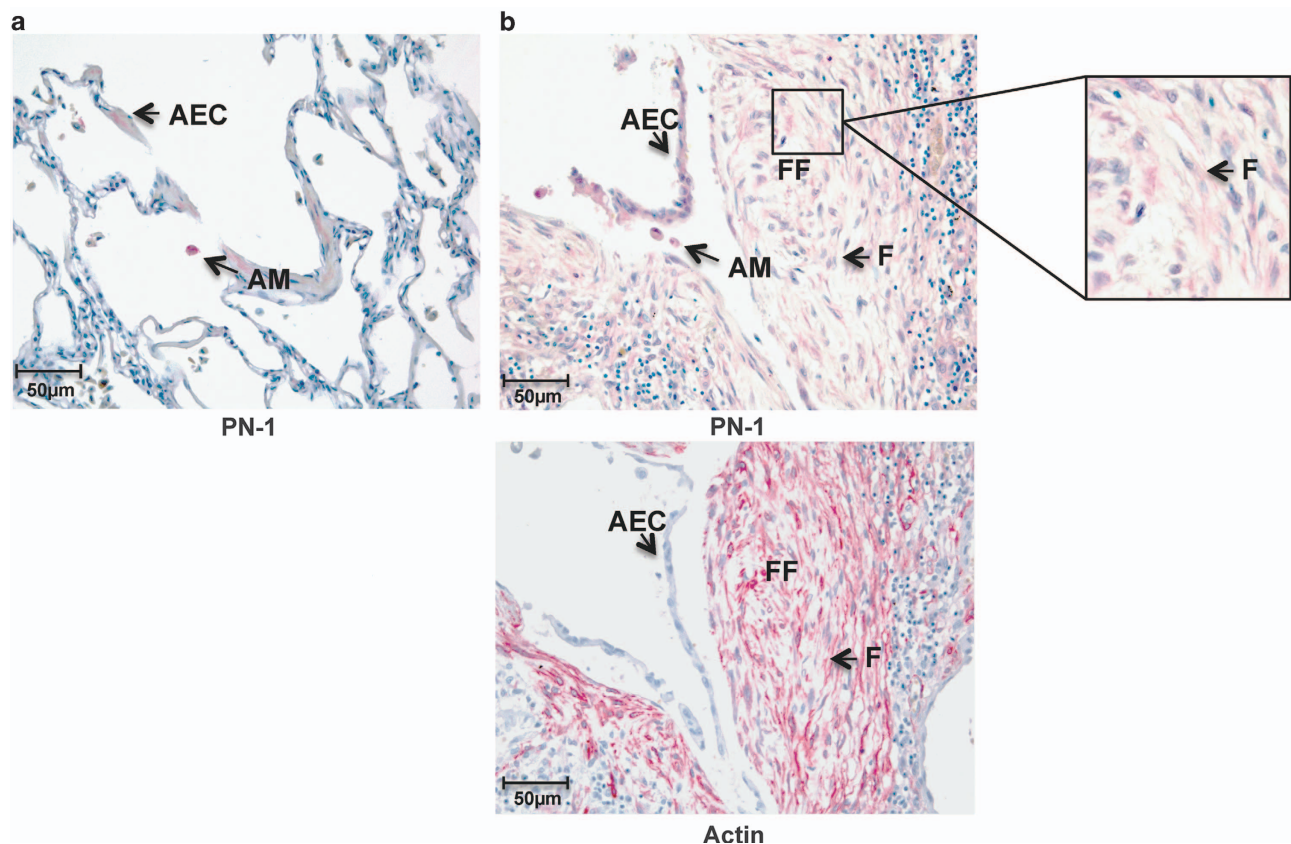


Figure 1 PN-1 localization in control lungs or lungs from patients with IPF. Human lung sections from controls (a) or patients with IPF (b) were analyzed by PN-1 immunostaining. Sections were incubated with a monoclonal anti-PN-1 antibody, revealed by using the Vectastain ABC-alkaline phosphatase kit. Macroscopic analysis allowed PN-1 localization in lung sections at magnifications × 20 and × 40. Alveolar macrophages (AM), fibroblasts (F) in a fibroblastic focus (FF), and alveolar epithelial cells (AEC) were identified by morphological analysis.

The following predesigned annealed siRNA were chosen for PN-1 silencing: sense sequence 5'-GGUUUCAAUCA-GAUUGAGTT-3' and antisense sequence 5'-CACAAUCU-GAUUGAAAACCTT-3'. The predesigned annealed irrelevant siRNA from Eurogentec was used as negative control. The duplexes were introduced into confluent IPF fibroblasts using lipofectamine reagents according to the manufacturer's instructions (Invitrogen/Life Technologies SAS). Cells were assayed after 96 h of transfection to verify PN-1 extinction and quantify collagen I and fibronectin expression.

Statistical Analysis

Results are shown as means \pm s.e.m. The Student's *t*-test (cell culture experiments) or the Mann and Whitney's test (patients experiments) were used to analyze all experimental results, and $P < 0.05$ was considered significant.

RESULTS

PN-1 Localization in Normal and Pathological Human Lung

Control lung sections showed an immune-reactivity for PN-1 in alveolar inflammatory cells, in particular in alveolar macrophages (AM) and in alveolar epithelial cells (Figure 1a). PN-1 immunostaining in lung samples from patients with IPF demonstrated additional prominent immunolocalization of PN-1 in the fibroblastic focus (FF) clearly associated with fibroblasts as revealed by the positive α -smooth muscle actin staining (Figure 1b).

Quantification of PN-1 mRNA in fibrotic and control lung tissues showed a two-fold increase of PN-1 expression in lung tissues from patients with IPF compared with controls (Figure 2a).

Immunoblot analysis of human BAL fluid (BALF) from patients with IPF revealed a single band of 45 kDa corresponding to native PN-1 that was weakly detectable in controls (Figure 2b). Additional high-molecular-weight bands could be observed in BALF only in non-reducing conditions, indicating the presence of complexes formed between PN-1 and other molecules via disulfide bridges. The dissociation of these higher bands under reducing conditions clearly demonstrates that they did not correspond to PN-1-protease complexes formed via the classical acyl-enzyme bond.

PN-1 Expression in Human Control and Fibrotic Pulmonary Fibroblasts

As shown in Figure 3a, PN-1 mRNA was detected in control cultured pulmonary fibroblasts at confluence. A significant increased expression of PN-1 mRNA (3.5-fold increase) was observed in fibroblasts cultured from patients with IPF (IPF fibroblasts), supporting the data obtained with the lung tissue extracts. PAI-1 was also expressed by pulmonary fibroblasts, and its expression was also increased in IPF fibroblasts (Figure 3a). The profibrotic phenotype of IPF fibroblasts was supported by the markedly increased expression of messengers of MEC components, such as collagen 1 α 2 (2-fold increase), collagen 3 α 1 (4-fold increase), and fibronectin (4.5-fold increase) (Figure 3a).

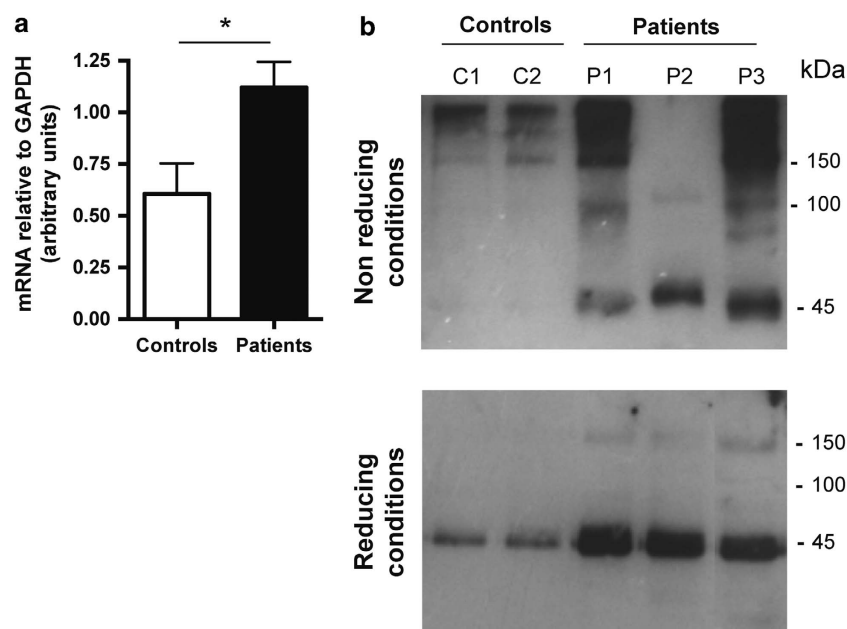


Figure 2 PN-1 is overexpressed in lung extracts and bronchoalveolar lavage (BAL) from patients with IPF. **(a)** PN-1 mRNA was extracted from lung tissue in controls and IPF patients, measured by qPCR and normalized with a housekeeping gene (GAPDH). Results are presented as means \pm s.e.m with $n = 7$ for controls and IPF patients ($*P < 0.05$ significantly different from controls). **(b)** BALs were analyzed by immunoblot using the monoclonal anti-PN-1 antibody in non-reducing and reducing conditions.

We can also observe an increased expression of PN-1 in IPF fibroblasts compared with control fibroblasts by immunoblot (Figure 3b), supporting the data obtained with the mRNA analysis. Indeed, the results showed that increased expression of PN-1 was largely detected in the CM of IPF fibroblast cultures, whereas little PN-1 was detected in the CM of control fibroblast cultures. PN-1 expressed by control fibroblasts remained essentially bound to cell surface because of the well-known high affinity of PN-1 to cell surface GAG. Altogether, these results indicate that when the fibroblast surface was saturated by PN-1, the excess of PN-1 expressed in fibrotic fibroblasts was released from the cell surface and found in the CM.

TGF- β Upregulates PN-1 Expression in Human Pulmonary Fibroblasts

The effect of the well-known profibrotic cytokine TGF- β on PN-1 expression was analyzed at both the mRNA and protein levels, by qRT-PCR and immunoblotting. Exposure of control fibroblasts to TGF- β induced the upregulation of PN-1 mRNA in a dose-dependent manner. The maximal

stimulatory effect was reached at 10 ng/ml TGF- β , PN-1 mRNA being increased by 11-fold compared with unstimulated fibroblasts (Figure 4a). In time course experiments, the upregulation of PN-1 messenger was maximal after 24 h of incubation with 10 ng/ml TGF- β (Figure 4b).

TGF- β also upregulated PN-1 protein expression by control fibroblasts in a dose (Figure 4c) and time (Figure 4d) dependent manner, the maximal stimulatory effect being observed at 10 ng/ml TGF- β for 24 h. Increased PN-1 was found in large amount in the CM from TGF- β -stimulated fibroblasts, indicating that PN-1 was secreted by TGF- β -stimulated fibroblasts as observed with IPF fibroblasts.

PN-1 from Fibrotic Fibroblasts Inhibits Thrombin

We examined whether increased PN-1 expressed by fibrotic fibroblasts was functional by investigating its ability to inhibit the amidolytic activity of purified thrombin. CM from unstimulated control fibroblasts had no effect on thrombin activity (Figure 5a). In contrast, thrombin residual activity dropped to $29 \pm 5\%$ and $42 \pm 3\%$ of its initial activity, when incubated with CM from TGF- β -stimulated and IPF

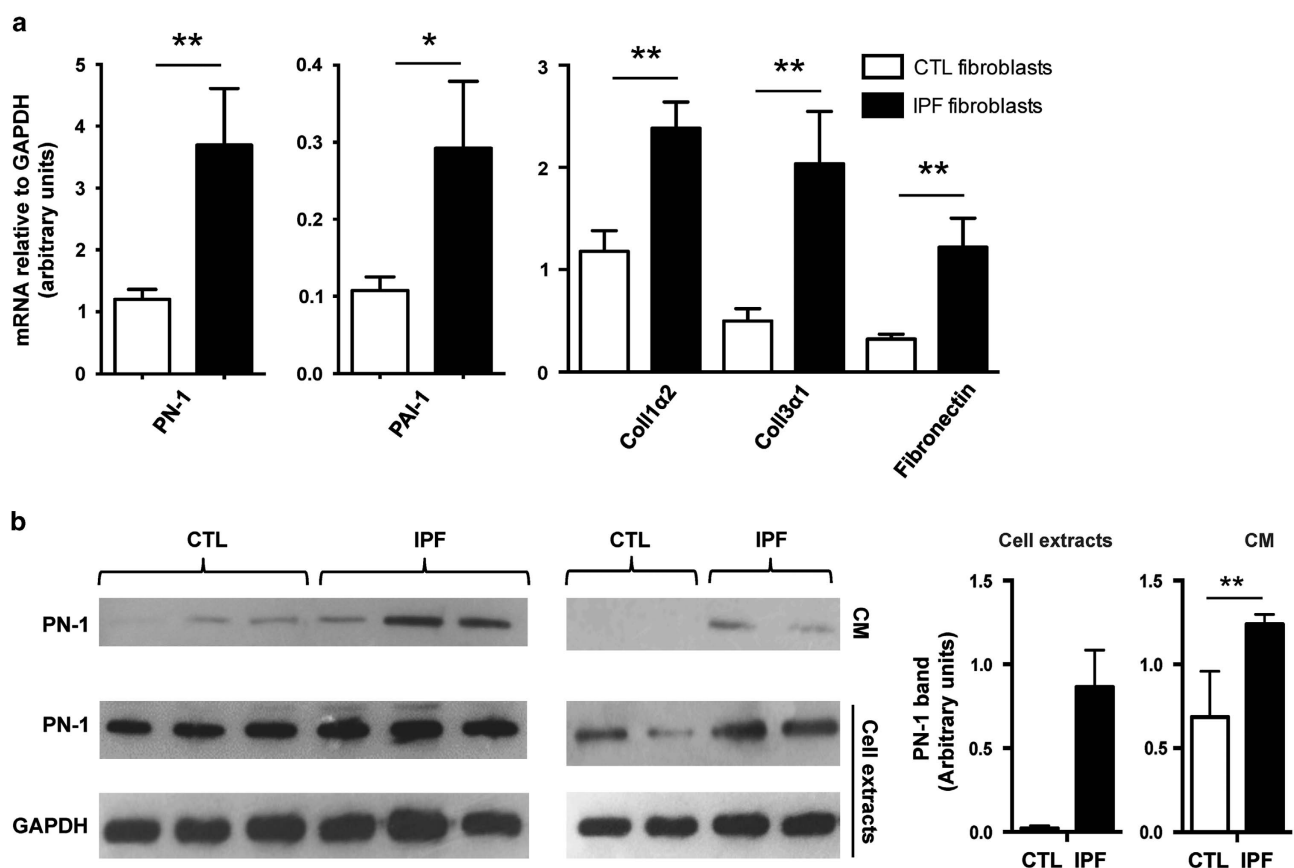


Figure 3 PN-1 is overexpressed in lung fibroblasts from patients with IPF. mRNA and protein were extracted from control (CTL) fibroblasts and IPF fibroblasts. (a) PN-1, PAI-1, collagen 1 α 2, collagen 3 α 1, and fibronectin mRNA were measured by qPCR and normalized with a housekeeping gene (GAPDH). Results are presented as means \pm s.e.m with $n = 4$ for CTL and IPF fibroblasts (** $P < 0.01$ and * $P < 0.05$ vs CTL fibroblasts). (b) PN-1 expression in cell extracts and conditioned media (CM) were analyzed by immunoblotting using anti-PN-1 and anti-GAPDH antibodies, and results were quantified by densitometric analysis. On the left, immunoblots are shown, and on the right, densitometric analysis of the corresponding results are shown as means \pm s.e.m with $n = 5$ for CTL and IPF. (** $P < 0.01$ vs control fibroblasts).

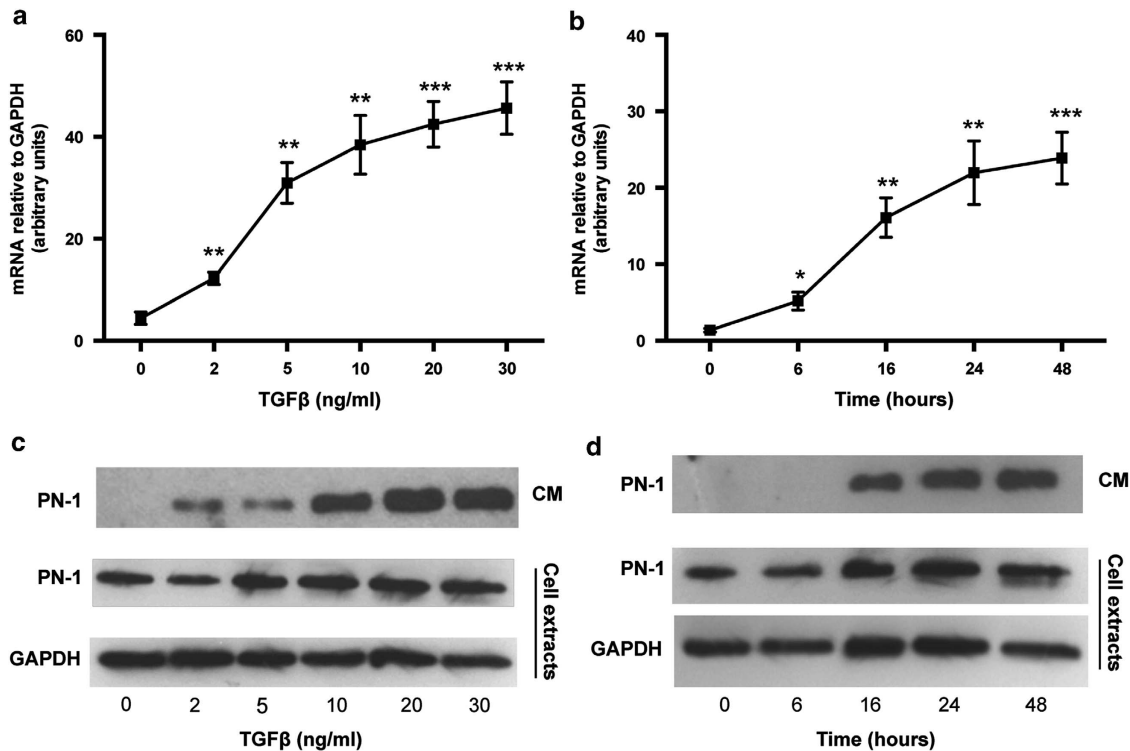


Figure 4 TGF- β upregulates PN-1 expression in control lung fibroblasts. mRNA and proteins were extracted from fibroblasts as described in Materials and Methods. (a, c) Fibroblasts were stimulated by increasing doses of TGF- β (0–30 ng/ml) for 24 h or (b, d) by 10 ng/ml TGF- β for 0–48 h. (a, b) PN-1 mRNA of was measured by qPCR (means \pm s.e.m; $n = 3$ independent experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs unstimulated cells). (c, d) Cell extracts and conditioned media (CM) were analyzed by immunoblotting using anti-PN-1 and anti-GAPDH antibodies.

fibroblasts, respectively, indicating the presence of a thrombin inhibitor in these CM (Figure 5a). To verify that this thrombin inhibitor was PN-1, the same experiments were performed in the presence of a blocking anti-PN-1 antibody. This blocking antibody significantly abolished thrombin inhibition by the CM derived from both IPF and TGF- β -stimulated fibroblasts. Such an effect was not observed with an irrelevant IgG or a blocking anti-PAI-1 antibody (Figure 5a). The same kind of result was obtained when analyzing thrombin catalytic activity directly on the cell surface of the different types of fibroblasts (Figure 5b). Indeed, both IPF and TGF- β -stimulated fibroblasts acquired a potent anti-thrombin effect that was reversed in the presence of the blocking anti-PN-1 antibody (Figure 5b). Together, these results indicate that the increase of PN-1 expression observed in fibrotic fibroblasts results in the accumulation of active PN-1 both at the cell surface and in the culture medium.

PN-1 Overexpression in Pulmonary Fibroblasts Induces Fibronectin Expression

To establish whether PN-1 could influence the expression of ECM proteins, we induced an overexpression of PN-1 in control fibroblasts by transient transfection with a vector containing the complete PN-1 coding sequence. A marked

increase in the expression of PN-1 was found compared with cells transfected with an empty vector (Figure 6a). Overexpression of PN-1 was accompanied by a two-fold increase in the expression of fibronectin, whereas it had no significant effect on the expression of collagen 1 α 2 and collagen 3 α 1 transcripts (Figure 6a). Conversely, a two-fold decrease in the expression of fibronectin was observed when PN-1 expressed by fibrotic fibroblasts from IPF patients was silenced by siRNA transfection (Figure 6b), whereas no change was observed in the expression of collagen 1 α 2 and collagen 3 α 1 transcripts.

PN-1 and the majority of the other serpins bind to their targets by using a substrate recognition sequence contained in an exposed reactive center loop. They also have a GAG binding site that causes the modulation by GAG of the activity of several serpins. To verify that fibronectin overexpression depended on the expression of functional PN-1, we transfected control fibroblasts with a vector containing, respectively, the PN-1 coding sequence with a mutated reactive center loop (RS mutant PN-1) or with a mutated GAG-binding site (HS mutant PN-1). Both mutated PN-1 molecules failed to significantly induce fibronectin overexpression (Figure 6a), indicating that both the antiprotease activity of PN-1 and its GAG-binding site are required for such an effect.

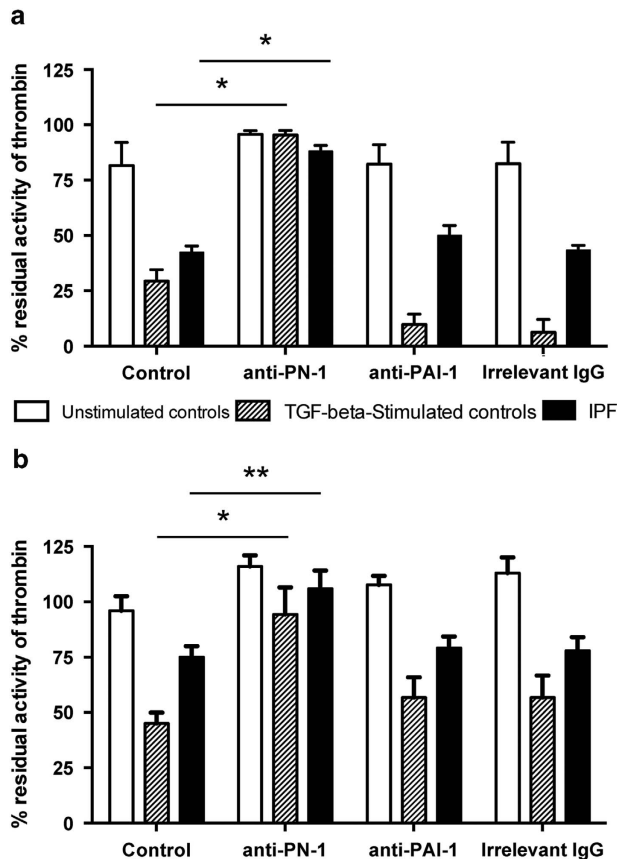


Figure 5 Inhibition of thrombin catalytic activity by PN-1 present in lung fibroblasts. Thrombin catalytic activity was measured after incubation (a) with conditioned media from unstimulated control fibroblasts, TGF- β -stimulated fibroblasts, or IPF fibroblasts or (b) on cell surface of unstimulated control fibroblasts, TGF- β -stimulated fibroblasts, or IPF fibroblasts. Incubations were performed in the presence or absence of a blocking anti-PN-1 IgG or anti-PAI-1 IgG or an irrelevant IgG. Results are presented as means \pm s.e.m of three independent experiments, each performed in triplicate (* P < 0.05; ** P < 0.01 vs respective controls).

DISCUSSION

IPF is characterized not only by an accumulation of excess fibrous material in the lung resulting from an excessive production and deposition of ECM on the one hand but also by decreased proteolysis and clearance of ECM on the other hand. The tissue balance of protease and antiprotease activities is therefore a cornerstone of IPF pathogenesis. The roles of plasminogen, plasminergic enzymes, and their regulators in the development of IPF have been well documented. High levels of PAI-1 have been observed in IPF and are associated with poor clinical outcomes.^{2,19} We demonstrated in this study that PN-1, another serpin, was also abnormally highly expressed in the fibrotic lung tissue and detected in BALF from patients with IPF. Until now, no data were available concerning the role of PN-1 in the pulmonary fibrosis. PN-1 is the most potent tissue inhibitor of thrombin. Our results clearly showed that both PN-1 present on the fibroblast

surface and PN-1 secreted by lung fibroblasts were able to block thrombin activity, whereas PAI-1 had no such inhibitory effect, confirming that PAI-1 is a weak inhibitor of thrombin.²⁰ Such data suggest that the regulation of thrombin, and therefore coagulation in the lungs, might be ensured by PN-1 rather than PAI-1. However, the respective implications of PN-1 and PAI-1 in the pathogenesis of IPF remain unknown. If degradation of ECM and fibrinolysis are the dominant processes involved in lung remodeling occurring during IPF, PN-1 and PAI-1 should exhibit complementary roles. In contrast, if fibrin formation induced by thrombin and pleiotropic cellular effects of thrombin are other dominant processes, therefore PN-1 should play a major role in the pathogenesis of IPF.

In the normal situation, BALF is known to exhibit fibrinolytic activity due to the presence of uPA. The role of uPA is to clear fibrin formed after extravasation of plasma proteins into the alveolar space.²¹ During IPF, increased levels of inhibitors, in particular PAI-1, suppress this fibrinolytic activity.² Our data show, for the first time, that there is also an abnormal high level of PN-1 in BALF from patients with IPF. PN-1 has been previously shown to limit fibrinolysis within a blood clot via its ability to inhibit plasminergic enzymes.²² In the blood, the regulation of the plasminergic system by serpins, such as PAI-1, PN-1, or α 2-antiplasmin, depends on their access to the fibrin clot. Here, in the lung, we are in a situation where these serpins may have complementary actions to regulate the plasminergic system taking place in the lung tissue.

In lung tissue from patients with IPF, PN-1 immunostaining was not only observed in fibroblasts but also in large immune cells, the AM. This is in agreement with previous data showing that PN-1 is expressed by blood monocytes and monocyte-derived macrophages.²³ However, a major part of PN-1 immunostaining was detected in lung fibroblasts, in particular in fibrotic areas. PN-1 is known to be expressed by fibroblasts from many different tissue types, including foreskin fibroblasts,⁶ skin fibroblasts,⁸ or mouse embryonic fibroblasts.²⁴ In human foreskin fibroblasts, PN-1 expression has been shown to be upregulated by interleukin-1.²⁵ We showed here that TGF- β , a potent profibrogenic cytokine, could also upregulate PN-1 expression in lung fibroblasts. Such upregulation of PN-1 expression induced by TGF- β has been shown in many different cultured cells, including neuroblastoma,²⁶ myotubes,²⁷ renal epithelial cells or cortical collecting duct cells,²⁸ and vascular smooth muscle cells.²⁹ PAI-1 is also overexpressed by pulmonary fibroblasts stimulated by TGF- β ,³⁰ and functional analysis of both PAI-1 and PN-1 promoters have identified a regulatory region for TGF- β induction, involving Smad complex binding.²⁹ The possibility that upregulation of PN-1 expression is a common process in fibrosis whatever the tissue affected remains to be determined.

PN-1 was present not only in the CM from fibroblast cultures but also in cell extracts. In contrast, PAI-1 is a

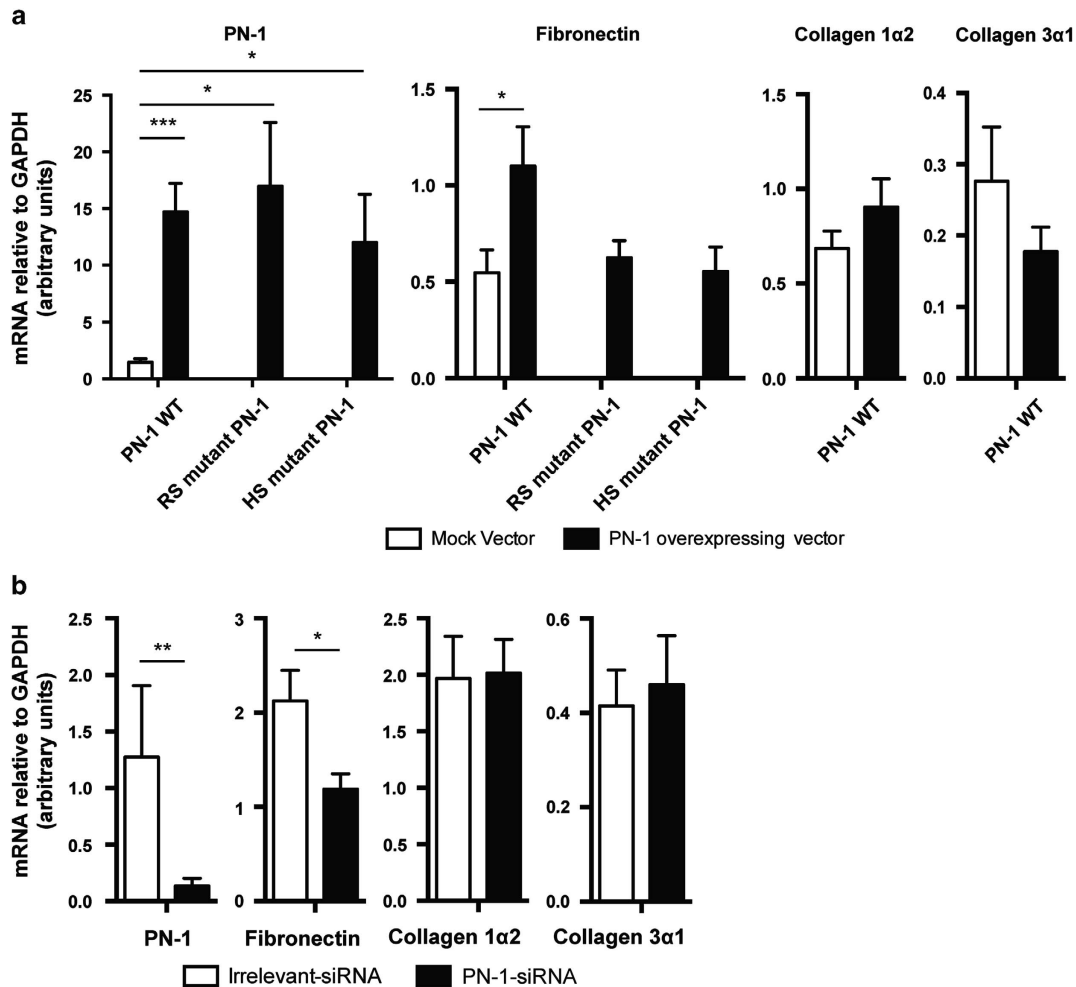


Figure 6 Modulation of PN-1 expression in lung fibroblasts regulates fibronectin expression. Control fibroblasts were transiently transfected with a WT PN-1 overexpressing vector or mutated PN-1 overexpressing vectors: reactive center loop mutant (RS) or GAG binding site mutant (HS). Negative controls were performed with a mock vector. (a) PN-1, fibronectin collagen 1α2, and collagen 3α1 mRNA were analyzed by qPCR and normalized with the housekeeping gene (GAPDH). Results are presented as mean ± s.e.m of four independent experiments, each performed in duplicate (* $P < 0.05$; *** $P < 0.001$ vs transfection with the mock vector). (b) IPF fibroblasts were transfected with irrelevant small interfering RNA (siRNA) or siRNA for PN-1 (PN-1-siRNA). PN-1, collagen 1α2, collagen 3α1, and fibronectin mRNA were analyzed by qPCR and normalized with GAPDH. Results are presented as mean ± s.e.m of four independent experiments, each performed in duplicate (* $P < 0.05$; ** $P < 0.01$ vs transfection with the mock vector).

protein directly secreted into the culture medium and therefore poorly detectable in cell extracts. Such differences in cellular localization between PAI-1 and PN-1 can be explained by the fact that PN-1 has a higher affinity for GAG than PAI-1. Indeed, PN-1 has been shown to remain essentially associated with cell surface GAG and to be released from the cell surface by polysaccharides, such as heparin³¹ or fucoidans.³² Thus, in physiological conditions, PN-1 and PAI-1 are present in different compartments and therefore may target different proteases. We found that part of the PN-1 induced by TGF-β-stimulated and IPF fibroblasts was detected in CM in large amounts because of a saturation of the fibroblast surface under these fibrotic conditions. These variations in the localization of PN-1 suggest that its role may become

important under conditions of stress, as here under fibrotic conditions.

The simultaneous increased expression of PN-1 and ECM proteins in TGF-β-stimulated and IPF fibroblasts raised the question of a potential direct modulation of ECM production by the abnormally high level of PN-1. We indeed showed that upregulation of PN-1 expression induced by transient transfection in control pulmonary fibroblasts was accompanied by an upregulation of fibronectin expression, and conversely PN-1 silencing induced by transfection with a PN-1-siRNA in fibrotic pulmonary fibroblasts from IPF patients was accompanied by a downregulation of fibronectin expression. These results suggest that PN-1 upregulation is not only a consequence of fibrosis but also contributes to fibronectin accumulation. No effect on fibronectin expres-

sion was observed when using mutated forms of PN-1, confirming a direct relationship between PN-1 and fibronectin expression in fibrotic fibroblasts, and that PN-1 requires both its antiprotease activity and its capacity to bind GAG to affect fibronectin expression. Upregulation of PN-1 expression has previously been shown to induce increased collagen transcript levels in mouse embryonic fibroblasts, but no data were provided for fibronectin transcripts.⁸ In contrast, our results did not show any significant change for collagen expression in lung fibroblasts overexpressing PN-1. We can assume that, according to the studied tissue type, PN-1 overexpressed by fibroblasts may be involved in the upregulation of certain MEC proteins. PN-1 may induce different signaling pathways, leading to a differential regulation of ECM proteins, depending on the studied cell type.

PN-1 has been shown to inhibit furin,³³ an ubiquitous proprotein convertase involved in the proteolytic processing of a wide range of precursor proteins, including adhesion molecules and various metalloproteinases known to be involved in ECM degradation.³⁴ Therefore PN-1 may also have a role in the modulation of ECM expression by its capacity to inhibit such regulatory proteases.

Our data confirm that PN-1 has a role in lung homeostasis. PN-1 has indeed recently been evaluated as a susceptibility gene for another devastating lung disease: chronic obstructive pulmonary disease (COPD).^{10,35} Some PN-1 variants have been associated with lung function among patients with COPD and asthma.³⁶ We thus propose that PN-1 is a new important player in pulmonary fibrosis and hypothesize that the biological activity of PN-1 could be of importance in the progression of the pathology. Such a role could be deleterious, regarding the impact of PN-1 on the induction of ECM proteins such as fibronectin or, in contrast, beneficial in view of the impact of PN-1 on thrombin activity. The novel identification of PN-1 as a new player in IPF development might lead to a better understanding of the lung remodeling occurring during this disease and thus be helpful for designing new therapeutic approaches.

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AUTHOR CONTRIBUTIONS

DF and LV contributed to acquisition, analysis and interpretation of data; JM-S carried out immunohistochemical experiments and, with BC, contributed to material tools and tissue specimen; MJ-P and VA revised the manuscript; M-CB contributed to conception of experiments, design of the study, interpretation of data, drafting and revising the article. All authors have approved the submitted manuscript.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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