Role of thrombin and its major cellular receptor, protease-activated receptor-1, in pulmonary fibrosis

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Abstract

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders and is characterized by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium. There is increasing evidence from a number of studies that activation of the coagulation cascade, with the resultant generation of coagulation proteases, plays a central role in fibrotic lung disease that is associated with acute and chronic lung injury. Consistent with this finding, levels of thrombin are increased in bronchoalveolar lavage fluid from patients and in animal models of this disorder. In addition to its classical role in blood coagulation, thrombin exerts a number of proinflammatory and profibrotic cellular effects in vitro that are critically important in tissue repair processes. These cellular effects are predominantly mediated via proteolytic activation of the major thrombin receptor protease-activated receptor-1 (PAR-1). This has led us to hypothesize that the procoagulant and the downstream cellular effects of thrombin, which are initiated following receptor activation, may be important in promoting tissue fibrosis in vivo. To examine this hypothesis, we assessed the effect of a direct thrombin inhibitor in bleomycin-induced pulmonary fibrosis in rats. Immunohistochemical studies showed that expression of thrombin and PAR-1 in lung tissue increased dramatically after intratracheal instillation of bleomycin, compared with saline-treated animals. After bleomycin instillation, there was a doubling in the amount of lung collagen after 14 days, which was preceded by elevations in \( \alpha_1(I) \) procollagen and connective tissue growth factor (CTGF) mRNA levels. However, when bleomycin-treated animals concurrently received a continuous infusion of a direct thrombin inhibitor at an anticoagulant dose, lung collagen accumulation in response to bleomycin was attenuated by up to 40%. Furthermore, \( \alpha_1(I) \) procollagen and CTGF mRNA levels were also significantly reduced in these animals. These findings confirm that thrombin is a key mediator in the pathogenesis of this condition and suggest that the cellular effects of thrombin may be critically important in promoting lung collagen accumulation in this experimental model of pulmonary fibrosis. Targeting the profibrotic effects of coagulation proteases warrants further evaluation as a potential therapeutic strategy for fibrotic lung disease.

Introduction

The prime function of the coagulation cascade is to generate a stable, insoluble, cross-linked fibrin blood clot to promote haemostasis at sites of vascular injury. The formation of the provisional clot, which comprises aggregated platelets enmeshed by fibrin, is critically dependent on the action of thrombin and is generated after the stepwise activation of coagulation proteases via two highly regulated, independent pathways: the extrinsic and intrinsic systems. Briefly, the extrinsic pathway is the trigger mechanism for blood coagulation and is activated when Factor VIIa comes in contact with extravascular tissue factor following vascular injury. Tissue factor and Factor VIIa contribute to the ‘extrinsic tenase’ complex, which activates Factor X, at the point of convergence with the intrinsic pathway. The intrinsic pathway is the trigger mechanism for blood coagulation and is activated when Factor VIIa comes in contact with extravascular tissue factor following vascular injury. Tissue factor and Factor VIIa contribute to the ‘extrinsic tenase’ complex, which activates Factor X, at the point of convergence with the intrinsic pathway. The intrinsic pathway is triggered when blood comes into contact with negatively charged components of subendothelial connective tissue, such as collagen. The pathway is initiated by the kallikrein/kinin system and involves sequential activation of Factor XII, Factor XI and Factor IX. Factor IXa, together with Factor VIIIa, contributes to the ‘intrinsic tenase’ complex and activates Factor X. At this point of the cascade, the extrinsic and intrinsic pathways merge to trigger a set of reactions in the common pathway. Factor Xa then binds to a cell surface receptor called effector protease receptor-1 and forms the prothrombinase.
complex, which is responsible for the conversion of prothrombin into thrombin. Thrombin cleaves soluble fibrinogen into insoluble fibrin and also activates Factor XIII which cross-links the fibrin clot and also promotes platelet aggregation, resulting in the formation of stable haemostatic plugs.

**Role of the coagulation cascade in pulmonary fibrosis**

A number of investigators have shown that activation of the coagulation cascade is a common feature of fibrotic lung disease. For example, intra-alveolar accumulation of fibrin has been described for patients with pulmonary fibrosis [1–3], acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [4,5], a condition in which rapid fibroproliferation and matrix synthesis can lead to the development of extensive fibrotic lesions [6]. Furthermore, bronchoalveolar lavage fluid (BALF) from patients with ARDS, has been shown to contain tissue factor–Factor VII/VIIa complexes, which can activate Factor X, and trigger activation of the extrinsic coagulation cascade [5].

The first evidence that thrombin may be an important mediator in the pathogenesis of fibrotic disease in humans was provided by studies that demonstrated that levels of active thrombin are increased in BALF from patients with pulmonary fibrosis associated with systemic sclerosis [7,8]. Similar findings were also obtained in the bleomycin model of pulmonary fibrosis [9,10]. It has been proposed that excessive procoagulant activity in ARDS may be owing to reduced levels of the endogenous thrombin inhibitor, antithrombin III [11]. In addition, protein C activity, which indirectly controls thrombin generation by inactivating coagulation protease complexes within both the intrinsic and extrinsic systems, is reduced in patients with interstitial lung disease and is associated with abnormal collagen turnover in the intra-alveolar space [12].

Several human and animal studies have examined the effects of modulating the coagulation cascade in acute and chronic lung disease. Exogenous delivery of the highly specific direct thrombin inhibitor, hirudin, or antithrombin III, is protective in animal models of ALI [13–15]. In addition, heparin, which inhibits coagulation proteases by potentiating the formation of antithrombin III–serine protease complexes, leads to improved gas exchange in an animal model of ALI [16]. Finally, heparin has also been shown to attenuate bleomycin-induced pulmonary fibrosis in mice [17].

**Biological function of thrombin in tissue fibrosis**

When the first studies documented that activation of the coagulation cascade was a common feature of fibrotic lung disease, many of the biological effects exerted by thrombin were still unknown. Research performed over the last 10 years has shown that thrombin mediates a number of cellular responses that may play critical roles in tissue repair responses. Most of these cellular effects are mediated by a unique family of ubiquitously expressed cell-surface receptors called protease-activated receptors (PARs), which are activated by limited proteolysis rather than direct ligand binding [18]. Although four PARs have been characterized to date, PAR-1 has been shown to be the major receptor involved in mediating thrombin’s mitogenic, pro-fibrotic and pro-inflammatory effects *in vitro* [19–21]. After the interaction of thrombin with its receptors, most of its cellular effects are thought to be mediated via the induction and release of a host of secondary mediators [22]. Consistent with this hypothesis, we have shown recently that thrombin is a potent inducer of connective tissue growth factor (CTGF) by human lung fibroblasts via direct proteolytic activation of PAR-1 [23]. CTGF is also induced in response to transforming growth factor β1, the most potent fibrogenic mediator characterized to date [24]. Since both thrombin and transforming growth factor β1 induce CTGF production in fibroblasts *in vitro*, it has been suggested that CTGF may be a common downstream mediator responsible for mediating the profibrotic effects of both molecules (reviewed on pages 194–200 in this issue [25]).

**Thrombin and PAR-1 expression in experimental bleomycin-induced pulmonary fibrosis**

Recent studies in our laboratory have assessed the role of thrombin and its major cellular receptor, PAR-1, in bleomycin-induced pulmonary fibrosis. Immunohistochemical studies performed to examine the exact localization of thrombin in the lung have shown that there is very weak expression of thrombin on resident alveolar macrophages in the normal lung. However, after bleomycin instillation, expression of thrombin is markedly increased and predominantly localized to macro-
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phages in inflammatory and fibroproliferative foci, and to fibroblast-like interstitial cells (see Figure 1). The immunoreactivity of thrombin was maximal 6 days after bleomycin instillation, which is in accord with previous reports of peak thrombin levels [9,10] and procoagulant activity associated with alveolar macrophages in BALF of bleomycin-treated rats [26]. There are a number of potential mechanisms that may lead to increased expression of thrombin on macrophages and interstitial cells following bleomycin injury. For example, the active protease may leak into the alveolar space from the vascular compartment as a result of chronic activation of the coagulation cascade, following the extensive and continued endothelial injury caused by bleomycin. In addition, macrophages and fibroblasts have been shown to express tissue factor, the primary cell-surface initiator of the extrinsic coagulation cascade in vitro [27,28]. Furthermore, macrophages express membrane assembly sites for the generation of the prothrombinase complex [29], responsible for activating the intrinsic coagulation cascade. This means that these cells could accelerate membrane-dependent coagulation reactions and facilitate the local extravascular generation of thrombin, independent of classical blood coagulation. Indirect support that these effects occur in the human lung has come from studies showing that thrombin is present on the surface of normal human alveolar macrophages [30] and that expression of tissue factor on these cells is increased dramatically in patients with pulmonary fibrosis [31].

The immunohistochemical localization of PAR-1 after bleomycin instillation, similarly to thrombin, showed that positive staining was predominately associated with macrophages in interstitial inflammatory and fibroproliferative foci, but was also present on elongated spindle-shaped cells in these areas (Figure 1). PAR-1 was also consistently expressed on the bronchial epithelium in sham-treated lung tissue from rats used in this study, although there was no evidence of altered expression following saline or bleomycin instillation.

Figure 1
Thrombin and PAR-1 expression is increased in bleomycin-induced pulmonary fibrosis

(a) A section of rat lung, 6 days after intra-tracheal instillation of saline. There is only weak staining for active thrombin on resident macrophages. (b) A corresponding lung section after intra-tracheal instillation of bleomycin. There is extensive staining for active thrombin, which is predominantly associated with macrophages in inflammatory and fibroproliferative foci but also interstitial cells (arrows). (c) The immunohistochemical localization of PAR-1, 6 days after intra-tracheal saline instillation. PAR-1 is expressed consistently on the bronchial epithelium, but the lung parenchyma is negative. (d) A corresponding lung section from a bleomycin-treated animal at the same time point. The expression of PAR-1 increased dramatically in the lung parenchyma of these animals and was again predominantly associated with macrophages in inflammatory and fibroproliferative foci, but elongated spindle-shaped cells were also positive in these areas. Scale bar, 70 μm. Reproduced from [41] with permission. © (2001) American Society for Investigative Pathology.
Consistent with our immunohistochemical findings, PAR-1 mRNA levels were also increased in the lungs of bleomycin-treated rats, compared with animals that were given saline alone. In addition, we were able to show that primary cultures of rat lung fibroblasts grown from sham-treated rats expressed PAR-1 at the mRNA and protein level and that these receptors were functional in proliferation and CTGF gene expression studies. The combination of these in vivo and in vitro findings suggests that the well characterized PAR-1-mediated cellular effects of thrombin may play an important role in this model of pulmonary fibrosis.

**Direct thrombin inhibition attenuates bleomycin-induced pulmonary fibrosis**

Evaluation of the contribution of thrombin in this animal model showed that the typical doubling in total lung collagen in response to bleomycin instillation in drug-vehicle control animals was attenuated by up to 40% in animals given a direct thrombin inhibitor as a continuous subcutaneous infusion (Figure 2). It is likely that thrombin is acting via both its procoagulant effects, i.e. fibrin deposition, and cellular effects in this model of bleomycin-induced lung injury. Fibrin is thought to influence the tissue repair/fibrotic response by acting as a provisional matrix in combination with fibronectin, on which fibroblasts can proliferate and produce collagen [32]. There is also evidence that fibrin can act as a reservoir of fibrogenic growth factors and cytokines, which are released during fibrinolysis [33]. However, the recent observation that fibrinogen knockout mice are not protected from developing pulmonary fibrosis in response to bleomycin has challenged the role of fibrin in the pathogenesis of this condition [34].

Given the widespread expression of PAR-1 in the interstitial compartment of the lung after bleomycin-induced injury, we examined whether thrombin's numerous cellular effects may play an important role in this experimental model. We were able to show that the previously reported increases in CTGF and \( \alpha_1(1) \) procollagen mRNA levels in response to bleomycin-induced lung injury [35,36] were reduced dramatically in rats that were treated with the direct thrombin inhibitor UK-156406 (see Figure 3). Indirect support for the idea that UK-156046 may exert its protective effects following bleomycin instillation by interfering directly with thrombin-mediated fibroblast responses was also provided by in vitro studies showing that thrombin-induced effects (fibroblast proliferation and increased procollagen and CTGF mRNA levels) were almost completely abolished at UK-156406 concentrations that would be attained at the dose used in this study in vivo. Since there is increasing in vitro evidence that thrombin exerts its profibrotic effects via the upregulation of secondary mediators, it is tempt-

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**Figure 2**

**UK-156406 attenuates lung collagen accumulation in bleomycin-induced pulmonary fibrosis**

(a) The effect of a continuous subcutaneous infusion of UK-156406 on lung collagen accumulation at 14 days after a single intra-tracheal instillation of bleomycin (1.5 mg/kg). ***P < 0.01, denotes the significance of the increase in total lung collagen between bleomycin-treated animals (Bleo) and saline-treated controls (Sal). **P < 0.05, denotes the significance of the reduction in lung collagen accumulation in bleomycin-treated animals that were given UK-156406; n = 6. The results obtained are representative of three separate experiments.

(b) UK-156406 inhibits thrombin proteolytic activity by binding in its catalytic triad. This inhibitor can therefore block the procoagulant and cellular effects of the protease. Figure 2(a) is reproduced from [41] with permission. © (2001) American Society for Investigative Pathology.
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To speculate that the effects of thrombin on lung collagen accumulation may be mediated, at least in part, via a CTGF-dependent mechanism. Future studies, utilizing PAR-1 knockout mice, will be critical in unravelling the contribution of the cellular versus the procoagulant effects of thrombin in promoting lung collagen deposition in this model, and are currently ongoing in our laboratory.

**Figure 3**

UK-156406 attenuates CTGF and α(I) procollagen mRNA levels in bleomycin-induced pulmonary fibrosis

(a) Increases in lung CTGF and α(I) procollagen mRNA levels in bleomycin-treated rats compared with saline control animals. Samples were normalized for RNA loading based on densitometric quantification of 28S rRNA (n = 6). **P < 0.01**, denotes the significance of the increase in CTGF and α(I) procollagen mRNA levels in bleomycin-treated animals compared with control animals. *P < 0.05*, denotes the significance of the reduction in these levels in bleomycin-treated animals that were given UK-156406. (b) and (c) show phosphorimages of representative Northern blots for the 2.4 kb CTGF mRNA and the 5.8 kb and 4.8 kb α(I) procollagen mRNAs. The corresponding ethidium bromide stained 28S rRNA bands are also shown. Reproduced from [41] with permission. © (2001) American Society for Investigative Pathology.

**Summary and conclusion**

Our studies provide compelling evidence that modulation of the proteolytic activity of thrombin, by direct thrombin inhibition, reduces lung collagen accumulation in the bleomycin-model of pulmonary fibrosis. A recent study, which showed that systemic administration of activated protein C also attenuates bleomycin-induced pulmonary fibrosis in mice, adds further authority to the observation [37]. The mechanistic studies performed in the present study, combined with the recent observation that fibrinogen-null mice are not protected from bleomycin-induced lung injury [34], add support to the hypothesis that the profibrotic receptor-mediated effects of thrombin may play a very important role in the pathogenesis of bleomycin-induced pulmonary fibrosis.

Activation of the coagulation cascade is a feature of a number of lung disorders associated with inflammation and excessive deposition of extracellular matrix proteins including idiopathic pulmonary fibrosis [1–3], pulmonary fibrosis associated with systemic sclerosis [7,8], ALI/ARDS [5] and more recently, airway remodelling in asthma [38]. Our findings suggest that modulation of the coagulation cascade, and more specifically, the profibrotic effects of coagulation proteases, warrant further evaluation as potential therapeutic strategies for the treatment of these disorders. In addition, PAR-1 antagonists, blocking antibodies and antisense oligonucleotides, which are currently being developed as potential antithrombotic agents [39,40], may represent an even more attractive therapy in the future, since they will selectively interfere with the profibrotic effects of thrombin, whilst avoiding potential haemostatic complications associated with direct proteolytic inhibition of coagulation proteases.

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**References**


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