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REVIEW

Procoagulant signalling mechanisms in lung inflammation and fibrosis: novel opportunities for pharmacological intervention?

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There is compelling evidence that uncontrolled activation of the coagulation cascade following lung injury contributes to the development of lung inflammation and fibrosis in acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and fibrotic lung disease. This article reviews our current understanding of the mechanisms leading to the activation of the coagulation cascade in response to lung injury and the evidence that excessive procoagulant activity is of pathophysiological significance in these disease settings. Current evidence suggests that the tissue factor-dependent extrinsic pathway is the predominant mechanism by which the coagulation cascade is locally activated in the lungs of patients with ALI/ARDS and pulmonary fibrosis. Whilst, fibrin deposition might contribute to the pathophysiology of ALI/ARDS following systemic insult; current evidence suggests that the cellular effects mediated via activation of proteinase-activated receptors (PARs) may be of particular importance in influencing inflammatory and fibroproliferative responses in experimental models involving direct injury to the lung. In this regard, studies in PAR_1 knockout mice have shown that this receptor plays a major role in orchestrating the interplay between coagulation, inflammation and lung fibrosis. This review will focus on our current understanding of excessive procoagulant signalling in acute and chronic lung injury and will highlight the novel opportunities that this may present for therapeutic intervention.
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Keywords: coagulation cascade; thrombin; proteinase-activated receptor; pulmonary fibrosis; acute lung injury; fibroblast

Abbreviations: ALI, acute lung injury; APC, activated protein C; ARDS, acute respiratory distress syndrome; CCL2, chemokine (C–C motif) ligand 2; CTGF, connective tissue growth factor; FVIIa, factor VII (activated); FXa, factor X (activated); IPF, idiopathic pulmonary fibrosis; KO, knockout; PAI-1, plasminogen activator inhibitor-1; PAR, proteinase-activated receptor; PDGF, platelet-derived growth factor; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TGFβ, transforming growth factor-beta

Overview: coagulation and fibrinolysis

The activation of the coagulation cascade is one of the earliest events initiated following tissue injury. The prime function of this complex and highly regulated proteolytic system is to generate insoluble, crosslinked fibrin strands, which bind and stabilize weak platelet haemostatic plugs, formed at sites of tissue injury. The formation of this provisional clot is critically dependent on the action of thrombin, and is generated following the stepwise activation of coagulation proteinases via the *extrinsic* and *intrinsic* systems (reviewed in Mann *et al*., 2003). *In vivo*, the activation of the coagulation cascade is initiated via the extrinsic pathway. Under normal circumstances, blood is not exposed to tissue factor (TF). However, upon tissue injury, exposure of plasma to TF expressed on non-vascular cells or on activated endothelial cells results in the formation of the TF-activated factor VII (FVIIa) complex. The TF–FVIIa complex subsequently catalyses the initial activation of FX to activated factor X (FXa) and FIX to activated factor IX. FXa in association with activated factor V catalyses the conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin, the main constituent of a clot. This mechanism is felt to generate only limited amounts of thrombin. Sustained coagulation is achieved when thrombin synthesized through the initial TF–FVIIa–FXa complex catalyses the activation of FXI, FIX, FVIII and FX. In this manner, the intrinsic pathway is activated, leading to the sustained generation of thrombin and blood coagulation. The extrinsic pathway is therefore paramount in initiating coagulation via the activation of limited amounts of

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thrombin, whereas the intrinsic pathway maintains coagulation by the dramatic amplification the initial signal.

The coagulation cascade is tightly controlled by both negative feedback mechanisms, as well as by circulating and locally produced endogenous *anticoagulants*. The extrinsic pathway is mainly controlled by TF pathway inhibitor (TFPI), which inactivates TF–FVIIa complexes after binding to FXa. The intrinsic pathway is controlled by antithrombin, which inhibits thrombin and other serine proteases of the coagulation cascade in the presence of heparin. Other important physiological inhibitors include heparin cofactor II and protease nexin-1, which inhibit thrombin; α_2-macroglobulin and α_1-antitrypsin (also known as α_1-proteinase inhibitor), which inhibit thrombin and factors IXa, Xa and XIa; and protein Z-dependent protease inhibitor, member of the serpin superfamily of proteinase inhibitors that produces rapid inhibition of factor Xa in the presence of protein Z, procoagulant phospholipids and Ca^{++}. Finally, when thrombin binds to the endothelial cell surface receptor, thrombomodulin, it is converted from a procoagulant into an anticoagulant by activating protein C. Protein C activation is further enhanced by the endothelial cell surface receptor, endothelial cell protein C receptor. Activated protein C (APC), in conjunction with protein S, inactivates factors Va and VIIIa and thereby suppresses further thrombin generation.

Fibrinolysis is initiated when plasminogen is converted to plasmin by the proteinases, urokinase-type or tissue-type plasminogen activator. Plasmin subsequently cleaves fibrin into a range of fibrin degradation products. Fibrinolytic activity in the vasculature is largely under the control of tissue-type plasminogen activator, whereas extravascular fibrinolysis in the lung is controlled by urokinase-type plasminogen activator. The conversion of plasminogen to plasmin by tissue-type and urokinase-type plasminogen activators is regulated by the endogenous inhibitor, plasminogen activator inhibitor-1 (PAI-1). The fibrinolytic system is also influenced by the plasma glycoprotein, thrombin-activatable fibrinolysis inhibitor and protein C inhibitor (PCI). During fibrin degradation, plasmin exposes C-terminal lysine residues on the fibrin molecule to potentiate its clearance. Thrombin-activatable fibrinolysis inhibitor cleaves these residues, which therefore favours fibrin persistence. PCI on the other hand suppresses plasminogen activation and also blocks the activity of APC.

Activation of the coagulation cascade in acute lung injury

In the normal uninjured lung, the alveolar haemostatic balance is generally antithrombotic and pro-fibrinolytic. However, in both acute lung injury (ALI) and chronic lung diseases such as pulmonary fibrosis, this balance appears to be greatly shifted in favour of procoagulant and antifibrinolytic activity. This section will review this evidence, the underlying causes for this unbalance and its pathological significance. For ALI/acute respiratory distress syndrome (ARDS), this evidence has recently been reviewed (Ware *et al*., 2006) and will therefore be only touched upon briefly here.

Acute lung injury and ARDS are common, life-threatening conditions leading to acute respiratory failure. These conditions arise from a variety of local and systemic insults, of which sepsis, pneumonia and trauma are the most common causes. ALI/ARDS is characterized by diffuse alveolar damage leading to disruption of the alveolar capillary barrier, pulmonary oedema and neutrophilic inflammation. Extravascular intra-alveolar accumulation of fibrin, often evident as hyaline membranes lining the denuded alveolar surface, has long been recognized as a pathological hallmark of ALI/ARDS and it is well recognized that the coagulation cascade and the subsequent fibrinolytic pathway, responsible for clearing the fibrin clot, are disregulated in these patients (reviewed in Idell, 2003). The rapid development of interstitial and intra-alveolar fibrosis can lead to the obliteration of airspaces and accounts for respiratory death in up to 40% of patients with ARDS (Marshall *et al*., 1998).

There is good evidence that the TF-dependent extrinsic pathway is the predominant mechanism by which the coagulation cascade is locally activated in the lungs of patients with ALI/ARDS. TF–FVII procoagulant activity is increased in bronchoalveolar lavage fluid (BALF) from patients with ARDS (Idell *et al*., 1989) and a recent study employing immunohistochemical staining for TF in human lung tissue from patients with ARDS reported prominent TF staining on alveolar epithelial cells as well as intra-alveolar macrophages and hyaline membranes (Bastarache *et al*., 2007). Locally activated coagulation zymogens, in combination with leakage of plasma proteins (including fibrinogen) into the alveolar space, as a consequence of microvascular injury are thought to be responsible for the extensive deposition of intra-alveolar and interstitial fibrin (Günther *et al*., 2003). In terms of anticoagulant factors, levels of antithrombin are reduced in patients with ARDS, and increased TF levels are not matched by a similar increase in TFPI levels (Gando *et al*., 2003). Plasma and intra-alveolar protein C levels are also decreased in patients with ALI/ARDS (Ware *et al*., 2007). The alveolar epithelium has also recently been shown to express thrombomodulin and endothelial cell protein C receptor and is capable of activating protein C (Wang *et al*., 2007). The alveolar epithelium may therefore play an important role in modulating intra-alveolar coagulation. Inflammation or injury to the epithelium may further play a significant role in shifting this balance to a procoagulant state.

Anticoagulant drug intervention in patients with ALI/ARDS

Drug intervention studies with anticoagulants in animal models have provided strong support that excessive coagulation activity may be of pathological significance in ALI/ARDS. Extrapolating the importance of these findings to human disease pathogenesis remains a major challenge, as does the development of effective therapies aimed at interfering with uncontrolled procoagulant activity in these disease settings. Despite the overwhelming success of

anti-coagulant strategies in experimental models of ALI, clinical trials of anticoagulants (for example, antithrombin III, TFPI) in patients have been largely disappointing. In contrast, the results of a phase III, randomized, double blind, placebo-controlled, multicentre PROWESS trial of intravenous infusion of the anticoagulant, APC in severe sepsis (which included patients with ARDS) and subsequent reduction in mortality resulted in Food and Drug Administration approval for the use of recombinant human APC (drotrecogin alfa (activated), Xigris) in patients with severe sepsis (sepsis associated with acute organ dysfunction) who have a high risk of death. ARDS subgroup analysis was not presented, and so the effectiveness of this agent to protect the lung in ARDS remains to be determined. Risk of catastrophic bleeding complications is the most common serious concern associated with recombinant human APC therapy, and so each patient being considered for therapy has to be carefully evaluated and anticipated benefits weighed against potential risks associated with therapy. The continued use of this agent is currently the subject of an active debate. There are several ongoing trials to determine whether anticoagulant therapy will be beneficial in ALI; one study will compare TFPI with placebo in patients with pneumonia. There are also currently two studies comparing recombinant human APC with placebo in ALI/ARDS patients and patients with infectious ALI, respectively. The results of these trials are eagerly awaited (reviewed in Ware *et al*., 2006).

Activation of the coagulation cascade in pulmonary fibrosis

There is also increasing evidence that therapies targeting the coagulation cascade may prove useful for respiratory conditions associated with chronic or repetitive lung injury, including pulmonary fibrosis. Pulmonary fibrosis represents the end stage of a heterogeneous group of disorders, of known and unknown cause, in which excessive deposition of collagen and other extracellular matrix proteins within the pulmonary interstitium leads to obliteration of airspaces and progressive loss of lung function (Figure 1). There is good evidence that the coagulation cascade is activated in several fibrotic lung diseases, including systemic sclerosis (Hernandez-Rodriguez *et al*., 1995), idiopathic pulmonary fibrosis (IPF) (Imokawa *et al*., 1997), sarcoidosis and hypersensitivity pneumonitis (Günther *et al*., 2000). As is the case for ALI, current evidence suggests a major role for the TF-dependent extrinsic pathway as the main activator of the coagulation cascade in these conditions. TF expression is highly upregulated on type II pneumocytes and to some extent on alveolar macrophages, in close association with fibrin deposits in the lungs of patients with IPF and systemic sclerosis (Imokawa *et al*., 1997). Levels of active thrombin are increased in bronchoalveolar lavage fluid from patients with pulmonary fibrosis associated with systemic sclerosis (Hernandez-Rodriguez *et al*., 1995) and in pulmonary fibrosis associated with chronic lung disease of prematurity (Dik *et al*., 2003). Several other procoagulant factors (fibrinogen, factors VII and X) have also been identified in patients with intra-alveolar fibrosis associated with bronchiolitis obliterans organizing pneumonia/cryptogenic organizing pneumonia (Peyrol *et al*., 1990). There is also evidence that the protein C pathway is deficient in the lungs of patients with IPF and sarcoidosis as well as collagen vascular disease-associated interstitial lung disease (Kobayashi *et al*., 1998). Moreover, decreased protein C activation was found to be associated with abnormal collagen turnover in the intra-alveolar space of patients with these conditions (Yasui *et al*., 2000). This study, together with the observations that thrombin in bronchoalveolar lavage fluid from patients with systemic sclerosis, contributes to the mitogenic activity of this fluid for cultured lung fibroblasts; Ohba *et al*. (1994) and Hernandez-Rodriguez *et al*. (1995) provided some of the earliest evidence that the coagulation cascade might influence the development of fibrosis in these patients. Reviewing the current evidence that the coagulation cascade plays a pathophysiological role in patients with acute and chronic lung injury and the opportunities this provides for therapeutic intervention will be a focus of much of the remainder of this article.

Anticoagulants are effective in blocking experimentally induced lung fibrosis

Strategies targeting the coagulation cascade with direct and indirect anticoagulants have provided strong support for a

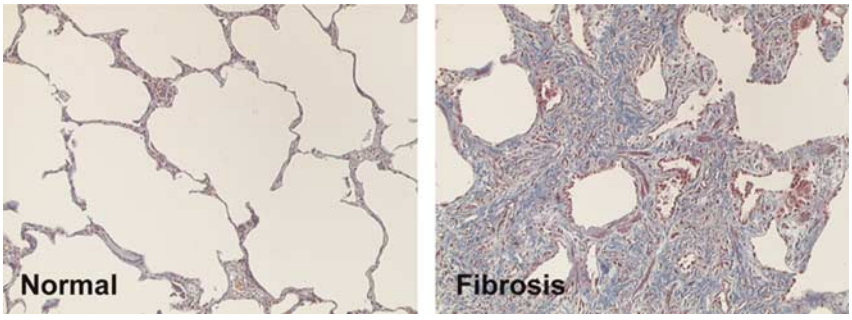

Figure 1 Alveolar architecture of normal and fibrotic lung. Images show the stark contrast in alveolar architecture in the normal and fibrotic lung. In the fibrotic lung, the open alveolar architecture is obliterated and replaced with dense fibrotic tissue. (Histology slides courtesy of Dr Robin McAnulty, Centre for Respiratory Research, University College London.)

causative role of the coagulation cascade in experimentally induced fibrosis. For example, our laboratory has shown that direct thrombin inhibition was highly effective at attenuating lung collagen deposition in bleomycin-induced lung fibrosis in rats (Howell *et al*., 2001). Similar findings have also been reported with the anticoagulant, APC instilled intratracheally in mice (Yasui *et al*., 2001) and with nebulized unfractionated heparin in rabbits (Günther *et al*., 2003). Although APC and heparin also exert anti-inflammatory effects, it seems likely that they may be acting, at least in part, via their anticoagulant properties in this model. More recently, intratracheal gene transfer of TFPI was also reported to decrease bleomycin-induced thrombin generation and pulmonary fibrosis in rats (Kijiyama *et al*., 2006). Taken together, these data support the notion that TF-mediated coagulation in the extravascular intra-alveolar space is of paramount importance and that anticoagulant therapy could be beneficial.

Anticoagulant therapy for patients with pulmonary fibrosis?

There have been few successful trials in pulmonary fibrosis, and so this condition remains largely untreatable (reviewed in Scotton and Chambers, 2007). However, the results of a recent non-blinded, randomized trial of 56 patients with IPF given prednisolone alone or prednisolone plus anticoagulation (Kubo *et al*., 2005) provides some support that targeting the coagulation cascade may improve outcome. In this study, the anticoagulant group had reduced mortality from acute exacerbations, with an overall significant increase in survival (63% survival at 3 years in the anticoagulant group versus 35% in the non-anticoagulant group). Although this was a small non-blinded study, and the exact mechanism of this beneficial effect is not known, it is one of the few studies describing a beneficial outcome on survival in an IPF clinical study reported on to date.

Fibrinolysis following lung injury

The prevailing balance between the pro- and anticoagulant states in the lung following injury is also affected by regulatory mechanisms that control the clearance of deposited fibrin (reviewed by Idell, 2003). There is compelling evidence that fibrinolysis is impaired in patients with both ALI/ARDS and pulmonary fibrosis. A number of human and animal studies have shown that levels of PAI-1 are increased in these conditions, thus favouring fibrin persistence (Olman *et al*., 1995). A recent study has further shown that levels of thrombin-activatable fibrinolysis inhibitor and PCI are increased in bronchoalveolar lavage fluid from patients with interstitial lung disease and may thereby contribute to intra-alveolar hypofibrinolysis associated with these conditions (Fujimoto *et al*., 2003).

The contribution of fibrin deposition to the development of experimental lung fibrosis has received considerable attention but remains a somewhat unresolved issue. Fibrin is thought to influence the fibrotic response in several ways. First, fibrin inhibits surfactant function and may thereby cause atelectasis (alveolar collapse). Second, according to the concept of 'collapse induration', by acting as a provisional matrix and reservoir of growth factors for fibroblasts and inflammatory cells, the fibrin matrix contributes to alveolar collapse and traction of remaining airspaces (honeycombing). Studies performed in experimental models using genetically modified mice in which the fibrinolytic capacity in the lung was either up- or downregulated support the notion that fibrin persistence may contribute to the development of bleomycin-induced fibrosis. Lung collagen accumulation is increased in mice overexpressing PAI-1 (favouring fibrin persistence) and is decreased in PAI-1 knockout (KO) mice (favouring fibrin clearance) (Eitzman *et al*., 1996). In further support for a role for fibrin in experimental fibrosis, aerosolization of urokinase-type plasminogen activator was highly effective in preventing bleomycin-induced lung fibrosis in rabbits (Günther *et al*., 2003). Similarly, the recent report that thrombin-activatable fibrinolysis inhibitor deficiency is associated with an attenuated response to bleomycin-induced lung fibrosis (Fujimoto *et al*., 2006) also supports the notion that fibrin persistence influences the subsequent fibrotic response. In contrast, the finding that fibrinogen KO mice are not protected from bleomycin-induced fibrosis (Hattori *et al*., 2000) suggests that fibrin *per se* may not be required for progression to fibrosis in bleomycin-induced lung fibrosis.

Proteinase-activated receptors: signalling receptors for coagulation proteinases

If fibrin is not required for experimental lung fibrosis, this begs the question as to how the coagulation cascade is causally involved in driving the fibrotic response. This problem was solved, at least in part, by the discovery of the proteinase-activated receptors (PARs) in the early 1990s (Vu *et al*., 1991).

The PARs belong to a subfamily of the seven transmembrane domain G-protein-coupled receptors and derive their name from their unique mechanism of activation involving the unmasking of a tethered ligand by limited proteolysis (Figure 2). Conformational changes induced following interaction of the tethered ligand with the second extracellular loop of these receptors initiate cell signalling via heterotrimeric G proteins. The PAR family comprises four members (PAR_1–PAR_4) and collectively, the proteinases of the coagulation cascade can target all four family members. For a summary of our current understanding on the major activators, expression patterns and pharmacology of the PARs, please see Table 1. All four PARs are expressed in the lung on a variety of resident cell types, as well as on cells that are recruited to the lung following injury. Thrombin is considered to be a major activator of PAR_1, PAR_3 and PAR_4; whereas FXa, either on its own or as part of the more potent TF–FVIIa–FXa ternary complex, activates either PAR_1 or PAR_2, depending on cell type and cofactor expression (reviewed in Coughlin 2005). Synthetic peptides corresponding to the tethered ligand sequence of PAR_1, PAR_2 and PAR_4 are capable of mimicking a number of cellular responses of

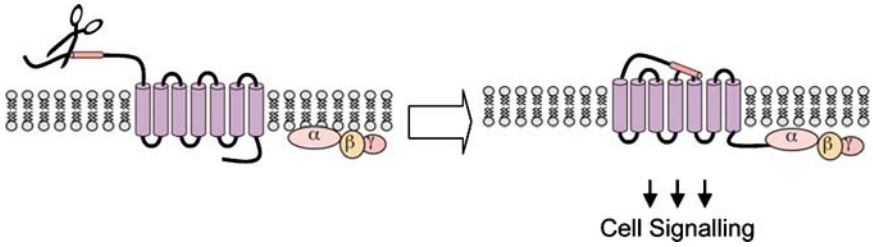

Figure 2 Activation of proteinase-activated receptors (PARs). Activation of PARs involves proteolytic cleavage of the N terminus leading to the unmasking of a tethered ligand, which subsequently binds to the second extracellular loop of the seven transmembrane domain receptor. This leads to a conformational change at the C terminus and recruitment of heterotrimeric G proteins.

endogenous proteinase activators. Whereas PAR_1, PAR_2 and PAR_4 act as signalling receptors, current evidence suggests that PAR_3 acts as a thrombin-docking receptor for efficient presentation of the proteinase to PAR_4 at low thrombin concentrations. Although there is little doubt that the PARs act as major signalling receptors for coagulation proteinases, it is important to point out that the PARs can also be activated by non-coagulation proteinases. In this regard, PAR_2 is a major substrate for trypsin as well as mast cell tryptase and has received considerable attention in the setting of both asthma and airway inflammation (reviewed in Moffatt *et al*., 2004).

PAR_1, the high-affinity thrombin receptor, was the first PAR to be cloned and fully characterized and has subsequently been shown to mediate thrombin's pluripotent cellular effects on numerous cell types. The clearest physiological role for PAR_1 is in the activation of platelets by thrombin, one of the key events involved in blood clotting. In addition, PAR_1 plays a central role in influencing a number of cellular responses that are central to the subsequent inflammatory and tissue repair programmes initiated following tissue injury (reviewed in Chambers, 2003). This receptor is currently a major drug target in the setting of thrombosis and cardiovascular disease (reviewed in Chackalamannil, 2006). The remainder of this article will discuss the evidence that PAR_1 may represent an attractive novel target for therapeutic intervention in the settings of both acute and chronic lung injury.

PAR activation and pro-inflammatory signalling

The role of PARs in promoting inflammation has been the subject of several excellent recent reviews (Coughlin 2005; Bunnett 2006) and will therefore only briefly be mentioned here. Extensive *in vitro* studies have revealed that activation of PAR_1 on numerous cell types, including among others fibroblasts, epithelial cells, monocytes/macrophages and vascular endothelial cells, leads to the induction and release of potent pro-inflammatory mediators and chemokines (Table 2). Similar potent pro-inflammatory effects have also been reported for factor Xa and TF–FVIIa–FXa complexes via both PAR_1- and PAR_2-dependent mechanisms and there is increasing evidence that these PAR-mediated pro-inflammatory responses may play significant roles in the context of a number of inflammatory conditions (reviewed in Bunnett, 2006). Activation of PAR_4, as well as PAR_2, with synthetic activating peptides has similarly been reported to lead to the release of interleukin-6 (IL-6), IL-8 and prostaglandin E_2 (PGE_2) by cultured bronchial epithelial cells (Asokananthan *et al*., 2002). A number of these mediators are potent inducers of TF expression, and so PARs may play a central role in perpetuating the interplay between coagulation and inflammation (Figure 3). Moreover, thrombin has also been reported to induce the expression of endothelial cell adhesion molecules, including P-selectin and intercellular adhesion molecule-1 (ICAM-1) *in vitro* and may therefore facilitate the recruitment of inflammatory cells via the production of chemokine networks and upregulation of adhesion molecule expression.

PAR_1 is also the major thrombin receptor expressed on microvascular endothelial cells, and activation of PAR_1 by thrombin promotes endothelial cell permeability and contraction *in vitro*. Direct intravenous infusion of thrombin increases pulmonary vascular permeability in experimental models (reviewed in Siflinger-Birnboim and Johnson 2003). An important role for PAR_1 in mediating these effects was provided by studies showing that thrombin-induced pulmonary microvascular permeability is abrogated in lung organ cultures from PAR_1 KO mice (Vogel *et al*., 2000). Widespread microvascular injury and leak are common features of ALI/ARDS and chronic fibrotic lung diseases and are thought to be a major mechanism leading to the extravasation of coagulation zymogens and intra-alveolar fibrin deposition (reviewed in Idell, 2003). Therapies targeting thrombin-dependent PAR_1 activation on the microvascular endothelium may therefore be of therapeutic value, although it is worth pointing out that activation of PAR_1 on the endothelium may also be cytoprotective in certain circumstances (Feistritzer *et al*., 2006).

PAR_1 is a major pro-fibrotic signalling receptor

The myofibroblast is the key effector cell in pulmonary fibrosis responsible for the production of the bulk of extracellular matrix proteins deposited within the pulmonary interstitium. This cell type is characterized by the *de novo* expression of contractile α-smooth muscle actin fibres and is thought to originate from three possible sources: expansion of the resident fibroblast pool; epithelial–mesenchymal transition or recruitment of circulating mesenchymal progenitor cells (fibrocytes) to sites of lung injury (reviewed in Scotton and Chambers, 2007). Thrombin exerts potent

Table 1 Human proteinase-activated receptor expression and pharmacology

	No. of amino acids	*High-affinity activating proteinases*	*Other activating proteinases*	*Tethered ligand sequence*	*Activating peptides*	*Antagonists*	*Inactivating proteinases*	*Expression in the lung*	*Cell types*
PAR1	425	Thrombin	TF/FVIIa/FXa complex, FXa, granzyme A, plasmin, trypsin IV, MMP-1, tissue kallikreins	R^{41}↑SFLLRN	SFLLRN-NH_2, TFLLR-NH_2	RWJ-5611, RWJ-58259, SCH 530348	Cathepsin G, neutrophil proteinase-3, elastase, chymase, Der p1	Airways, blood vessels, lung parenchyma	Endothelial cells, epithelial cells, fibroblasts, macrophages, mast cells, natural killer cells, neuronal cells, platelets, smooth muscle cells (airway and vascular), T cells
PAR2	397	Trypsin, tryptase, trypsin II, trypsin IV	TF/FVIIa/FXa complex, TF-FVIIa, FXa, matriptase/MT-SP1, proteinase-3Der p1, Der p3, Der p9, tissue kallikreins	R^{34}↑SLIGKV	SLIGKV-NH_2, SFLLRN-NH_2	None to date	Elastase, chymase	Airways, blood vessels, bronchial glands, lung parenchyma	Endothelial cells, epithelial cells, eosinophils, fibroblasts, mast cells, macrophages, monocytes, neuronal cells, neutrophils, platelets, smooth muscle cells (airway and vascular), T cells
PAR3	374	Thrombin	Trypsin, factor Xa	K^{38}↑TFRGAP	None known	—	Cathepsin G	Airways	Epithelial cells, fibroblasts, platelets, airway smooth muscle cells, T cells
PAR4	385	Thrombin, trypsin	Cathepsin G, tissue kallikreins	R^{47}↑GYPGQV	GYPGQV-NH_2, AYPGKF-NH_2	YD-3	Unknown	Airway, blood vessels, cardiovascular system	Endothelial cells, epithelial cells, smooth airway muscle cells, platelets, fibroblasts

Letters denote amino-acid sequences in one letter code; arrows denote cleavage site.
Abbreviations: Der p1, 3 and 9, house dust mite *Dermatophagoides pteronyssinus* proteinase 1, 3 and 9; FVIIa, activated factor VII; FXa, activated factor X; MMP-1, matrix metalloproteinase-1; MT-SP1, membrane-type serine protease 1; NH_2, amide; TF, tissue factor.

Table 2 Pro-inflammatory mediators released following PAR_1 activation

Cytokine/ chemokine	*Cell type*	*Reference*
IL-1β	Monocytes/macrophages	Naldini *et al.* (1998, 2002)
IL-2	T lymphocytes	Mari *et al.* (1994)
IL-6	Fibroblasts, epithelial cells, monocytes/macrophages, mast cells, smooth muscle cells	Sower *et al.* (1995), Cirino *et al.* (1996), Kranzhofer *et al.* (1996) and Naldini *et al.* (1998)
IL-8	Fibroblasts, epithelial cells, monocytes/macrophages	Ueno *et al.* (1996), Suk and Cha (1999), Ludwicka-Bradley *et al.* (2000) and Asokananthan *et al.* (2002)
PGE_2	Epithelial cells	Asokananthan *et al.* (2002)
CCL2/ MCP-1	Fibroblasts, endothelial cells, monocytes/ macrophages	Riewald *et al.* (2002), Bachli *et al.* (2003) and Colognato *et al.* (2003)
TNF-α	Monoctyes/macrophages	Naldini *et al.* (1998)

Table lists the major cytokines/chemokines released by cell types present in the lung.
Abbreviations: CCL2, chemokine (C–C motif) ligand 2; IL, interleukin; MCP-1, monocyte chemotactic protein 1; TNFα, tumour necrosis factor-alpha.

Figure 3 Proteinase-activated receptors (PARs) perpetuate the interplay between coagulation and inflammation. Activation of PARs leads to the induction of potent pro-inflammatory mediators, which are capable of inducing tissue factor expression. Tissue factor initiates the activation of the extrinsic coagulation pathway resulting in activation of PARs. The functional consequences in the injured lung include microvascular permeability, fibrin deposition, leukocyte recruitment and fibroblast activation.

pro-fibrotic effects *in vitro* by influencing fibroblast function and extensive *in vitro* studies involving selective PAR_1-activating peptides and cells derived from PAR_1-deficient mice have revealed that PAR_1 is the major signalling receptor involved in mediating the potent stimulatory effects of thrombin on lung fibroblast proliferation (Trejo *et al.*, 1996), extracellular matrix production (Chambers *et al.*, 1998) and fibroblast to myofibroblast differentiation (Bogatkevich *et al.*, 2001). We have further shown that PAR_1 is also the major receptor by which FXa stimulates lung fibroblast mitogenesis (Blanc-Brude *et al.*, 2005).

There is good evidence that the pro-fibrotic effects elicited following PAR_1 activation by thrombin and factor Xa are not mediated following PAR_1 activation directly but via the induction of a host of potent pro-fibrotic mediators. In terms of fibroblast mitogenic responses following PAR_1 activation,

this response is critically dependent on both the induction of platelet-derived growth factor-AA and upregulation of the platelet-derived growth factor α-receptor (Ohba *et al*., 1994; Blanc-Brude *et al*., 2001). Thrombin also induces the expression of platelet-derived growth factor-AA by other cell types, including macrophages (Tani *et al*., 1997) and blocking platelet-derived growth factor signalling has been successful in attenuating experimentally induced lung fibrosis (Rice *et al*., 1999; Yoshida *et al*., 1999). We and others have shown that PAR_1 activation also leads to the rapid and dramatic induction of connective tissue growth factor (CTGF) by cultured lung fibroblasts (Chambers *et al*., 2000) and epithelial cells (CTGF) (Riewald *et al*., 2001). CTGF has been shown to influence two discrete pro-fibrotic effects via two distinct domains: the N-terminal domain of CTGF mediates myofibroblast differentiation and collagen synthesis, whereas the C-terminal domain mediates fibroblast proliferation (Grotendorst and Duncan 2005).

More recently, another major mechanism by which activation of PAR_1 may promote fibrosis was uncovered by the observation that PAR_1 ligation can lead to the activation of latent transforming growth factor-beta (TGFβ) (Jenkins *et al*., 2006). TGFβ is one of the most potent pro-fibrotic mediators characterized to date and a major target in the context of developing novel therapeutic strategies for pulmonary fibrosis and other fibrotic conditions (reviewed in Scotton and Chambers 2007). The activation of latent TGFβ is a major rate-limiting step in the regulation of TGFβ bioavailability and involves the conversion of the latent precursor to its biologically active form through dissociation from the latency-associated peptide. In collaborative studies performed with our centre, Jenkins *et al*. (2006) have recently reported that PAR_1 ligation on epithelial cells leads to the activation of TGFβ via a αvβ6 integrin-dependent mechanism *in vitro* and that this mechanism contributes to TGFβ activity following bleomycin-induced lung injury. The significance of this finding and the potential role of CTGF downstream of PAR_1 activation in experimental lung fibrosis will be discussed in more detail in the next section. Finally, in the context of PAR_1 activation on epithelial cells, it has also been recently reported that activation of PAR_1 induces alveolar epithelial cell apoptosis *in vitro* (Suzuki *et al*., 2005). The importance of this finding in the context of lung injury and fibrosis remains to be established, but this may represent another potential mechanism by which excessive procoagulant signalling may exert deleterious effects.

Lessons from PAR KO mouse, PAR agonist and antagonist studies in experimental models of lung injury

Recent studies conducted in our laboratory using PAR_1 KO mice support a major role for PAR_1 in influencing inflammatory cell recruitment, microvascular leak, lung oedema and fibrosis in response to bleomycin-induced lung injury (Howell *et al*., 2005; Jenkins *et al*., 2006). The protection from bleomycin-induced lung inflammation and fibrosis in PAR_1 KO mice is associated with a reduction in the upregulation of the PAR_1-inducible mediators, chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemotactic protein 1/JE and CTGF. TGFβ lung levels are also reduced compared with correspondingly injured wild-type mice, but current *in vitro* data obtained using cultured lung epithelial cells indicate that thrombin/PAR_1 does not upregulate the expression of TGFβ directly, but as mentioned above acts at the level of activation of the latent TGFβ complex. The significance of this finding in experimental lung injury is supported by the observation that TGFβ signalling, as evidenced by nuclear phosphorylated Smad 2 immunostaining, is attenuated in PAR_1 KO mice compared with correspondingly injured wild-type mice (Jenkins *et al*., 2006). Once activated, TGFβ is a potent inducer of its own production *in vitro* and *in vivo* (Sime *et al*., 1997), and so the attenuated response in terms of TGFβ expression in PAR_1 KO mice following bleomycin-induced lung injury may be explained by a diminished ability of these mice to activate latent TGFβ. More recently, we have also obtained unpublished evidence that PAR_1 may promote TGFβ activation by lung fibroblasts via a non-integrin-mediated but thrombospondin-1-dependent mechanism, suggesting that PAR_1 may play an important role in controlling TGFβ bioavailability via several activation mechanisms.

The finding that CTGF expression is blunted in PAR_1 KO mice in response to bleomycin-induced lung injury is also of particular interest in light of our previous report that protection from bleomycin-induced lung fibrosis by direct thrombin inhibition is also accompanied by a blunted CTGF response (Howell *et al*., 2001). CTGF levels are increased in patients with fibrotic lung disease (Allen *et al*., 1999), but the mechanisms by which CTGF contributes to the development of lung fibrosis is currently poorly understood. Although CTGF exerts pro-fibrotic effects *in vitro* (Grotendorst and Duncan, 2005), adenoviral gene transfer of CTGF to the lung was shown to induce only a mild and transient fibrotic response, suggesting that CTGF is not a direct fibrogenic factor in this organ (Bonniaud *et al*., 2003). However, CTGF is capable of inducing fibrosis when co-administered with bleomycin in 'fibrosis-resistant' BALB/c mice, potentially by promoting a non-degradative environment (Bonniaud *et al*., 2004). The upregulation of CTGF following lung injury may therefore represent another potential mechanism by which PAR_1 may influence the subsequent fibrotic response. Finally, the diminished expression of CCL2/monocyte chemotactic protein 1/JE in PAR_1 KO mice following bleomycin injury is also of particular interest, in view of recent reports that CCL2 blockade and chemokine (C–C motif) receptor 2 deficiency (the main CCL2 signalling receptor) also lead to a blunted fibrotic response to both bleomycin and fluorescein isothiocyanate-conjugated-induced lung injury, potentially by influencing the recruitment of circulating fibrocytes (Moore *et al*., 2005). Interestingly, PAR_1 antagonism is also protective in experimental liver fibrosis, and stellate cells were found to upregulate CCL2 following PAR_1 activation *in vitro* (Fiorucci *et al*., 2004), so that the PAR1/CCL2 axis may be significant in a number of fibrotic conditions.

In other models of ALI such as during high-tidal-volume ventilation, intratracheal instillation of PAR_1-activating peptides (TFLLRN) increases lung oedema via the same αvβ6-dependent latent TGFβ activation mechanism

described above (Jenkins *et al.*, 2006). In contrast to these findings, in spontaneously breathing mice, intratracheal instillation of PAR_2 (SLIGRL)-, but not PAR_1-, activating peptides was sufficient to induce acute lung inflammation via a neuropeptide-dependent mechanism (Su *et al.*, 2005). Moreover, PAR_1 KO mice are not protected in the mouse model of systemic endotoxin-induced inflammation (endotoxaemia) (Pawlinski *et al.*, 2004). Current evidence suggests that multiple PARs (PAR_1–PAR_4) and fibrin formation together contribute to systemic endotoxin-induced inflammation (Pawlinski *et al.*, 2004; Camerer *et al.*, 2006). Taken together, these findings suggest that the contribution of PAR_1 to lung inflammation is likely to be highly dependent on the nature of the insult (bleomycin versus endotoxin) and also on whether the primary insult originates in the lung or in the systemic circulation.

Clinical implications for patients with pulmonary fibrosis: PAR_1 antagonists as novel agents for therapeutic intervention?

The protection of PAR_1 KO mice from lung oedema, inflammatory cell recruitment and the development of fibrosis in the bleomycin model of lung injury and fibrosis demonstrates that this receptor plays a central role in orchestrating the tissue response to lung injury. The finding from our and other laboratories that this receptor is highly upregulated on fibroblasts and macrophages within fibrotic foci in the lungs of patients with IPF (Howell *et al.*, 2001) and pulmonary fibrosis associated with systemic sclerosis (Bogatkevich *et al.*, 2005) is consistent with the notion that this receptor may also play a central role in the pathogenesis of human fibrotic lung disease. PAR_1 expression is upregulated in response to a number of pro-inflammatory and pro-fibrotic mediators (reviewed in Sokolova and Reiser, 2007), but the mediators responsible for controlling PAR_1 expression in the fibrotic lung are currently unknown. A recent report that autocrine production of COX-2-derived PGE_2 is responsible for downregulating the expression of PARs (PAR_1–PAR_3) following PAR_1 activation in cultured human lung fibroblasts (Sokolova and Reiser, 2007) is of particular interest in view of the compelling evidence that fibroblasts from patients with IPF are unable to upregulate COX-2 gene expression in response to various pro-inflammatory and pro-fibrotic stimuli (Keerthisingam *et al.*, 2001). This may provide a plausible explanation for the high levels of PAR_1 expression on these cells in IPF and further supports the notion that uncontrolled PAR_1 signalling may be of pathological significance in this disease setting. Recent unpublished data from our laboratory suggest that this receptor is also highly expressed on infiltrating macrophages and numerous fibroblasts present in fibrotic areas in lung tissue from patients with ALI/ARDS (Howell *et al.*, 2007). Finally, there is also accumulating evidence that procoagulant signalling may contribute to other respiratory conditions associated with remodelling, including airway remodelling in asthma (Terada *et al.*, 2004) and more recently also in chronic obstructive pulmonary disease (COPD) (Demeo *et al.*, 2006), as well as fibrosis in other organs such as the liver (Fiorucci *et al.*, 2004).

Progress in the development of PAR_1 antagonists

There is now increasing pre-clinical evidence that PAR_1-blocking strategies might prove useful for the treatment of fibroproliferative lung disease. It has been argued for some time now that such an approach may be safer in terms of bleeding complications compared with strategies aimed at directly interfering with the coagulation cascade as PAR_1 antagonists would allow selective inhibition of the potentially deleterious receptor-mediated cellular effects of coagulation proteinases, while preserving their essential role in fibrin formation. The development of PAR_1-specific receptor antagonists has been fuelled by the potential for such antagonists as antithrombotic/antiplatelet agents. The development of PAR_1 antagonists has been partially successful, but the clinical utility of these agents remains to be established. This section will briefly summarize recent progress in this field. For a recent review and further details, please see Chackalamannil (2006).

The early peptidomimetic PAR_1 antagonists were designed on the basis of the SFLLRN motif of the PAR_1-tethered ligand. Optimization of these antagonists led to the identification of the heterocycle-based peptide-mimetic of PAR_1 antagonists, indole-based RWJ-56110 and indazole-based RWJ-58259 (Zhang *et al.*, 2001; Damiano *et al.*, 2003). Both compounds inhibit thrombin-induced platelet aggregation with IC_{50} values of 340 and 370 nM. In the guinea pig model of *ex vivo* platelet aggregation, RWJ-58259 showed improved efficacy over RWJ-56110. RWJ-58259 completely inhibits thrombin-induced platelet aggregation at doses as low as $0.3\,mg\,kg^{-1}$ and this antagonist further displayed antithrombotic activity in a cynomolgus monkey arterial injury model. Studies with another related indole-based PAR_1 antagonist exerted protective effects in a rat model of liver fibrosis at a dose of $1.5\,mg\,kg^{-1}\,day^{-1}$ (Fiorucci *et al.*, 2004) and pre-clinical studies with RWJ-58259 are currently ongoing in our centre in both acute and chronic lung injury models.

Good PAR_1 affinity and promising activity in functional assays have also been obtained for non-peptide PAR_1 antagonists such as the pyrroloquinazoline analogues, such as SCH 79797. Promising data have been reported in thrombin-induced platelet aggregation assays ($IC_{50} = 3\,\mu M$) and other cell-based assays, and SCH 79797 was reported to limit myocardial ischaemia/reperfusion injury in rat hearts at an optimal dose of $25\,\mu g\,kg^{-1}$ i.v. (Strande *et al.*, 2007). However, this compound has been reported to be toxic for lung fibroblasts (Sokolova and Reiser, unpublished observations in Sokolova and Reiser, 2007). Potential off-target effects have also recently been reported (Di Serio *et al.*, 2007).

PAR_1 antagonists based on the core structure of the tetracyclic piperidine alkaloid, himbacine, from Australian magnolia trees have also recently been developed (Chackalamannil, 2006). The most potent PAR_1 antagonist in this series demonstrates excellent affinity (K_i of 4.3 nM), good oral bioavailability ($\sim$62%) and blocks platelet aggregation in the cynomolgus monkey model up to 70% at a dose of $3\,mg\,kg^{-1}$ (Chelliah *et al.*, 2007).

There are currently two pharmaceutical companies developing orally active PAR_1 antagonists in clinical trials in the setting of cardiovascular disease. A phase II trial of

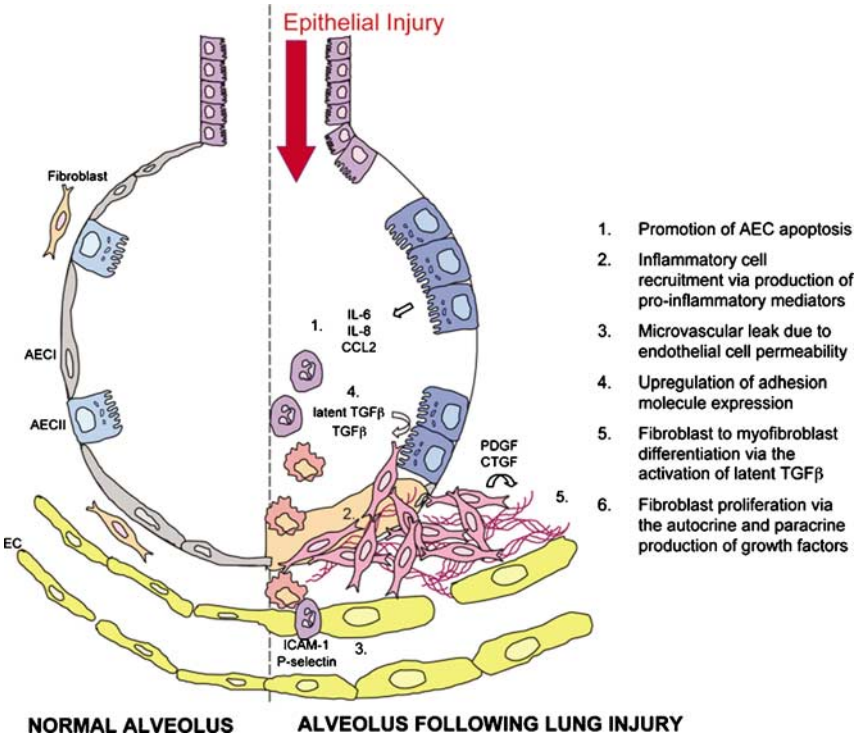

Figure 4 Role of proteinase-activated receptor 1 (PAR_1) in orchestrating the response to lung injury. The left-hand side shows the normal alveolar structure with flattened alveolar type I cells (AECI), sparse cuboid type II cells (AECII) and interstitial fibroblasts. Also shown is the microvasculature lined by endothelial cells. The right-hand side shows the effect of lung injury to the lung leading to denudation of the alveolar epithelium, hyperproliferation of AECIIs, inflammatory cell recruitment, microvascular permeability and proliferation and differentiation of fibroblasts. Activation of PAR1 on multiple cell types plays a central role in influencing a number of these processes (1–6). The *in vivo* role of all of these pathways remains to be established, but current experimental evidence supports a major role for PAR1 in promoting microvascular leak, inflammatory cell recruitment and fibroproliferative responses. Abbreviations: AECI, AECII, type I and II alveolar epithelial cells; EC, endothelial cell.

SCH 530348 in subjects undergoing non-emergent percutaneous coronary intervention (Study P03573AM1) has recently been completed (http://clinicaltrials.gov/show/NCT00132912), and two phase III trials to assess the effects of SCH 530348 in preventing heart attack and stroke in patients with atherosclerosis (TRA 2°P—TIMI 50) (Study P04737: http://clinicaltrials.gov/ct/show/NCT00526474) and in patients with acute coronary syndrome (TRA · CER) (Study P04736) have recently been announced (http://clinicaltrials.gov/ct/show/NCT00527943). The results of these trials are eagerly awaited and may hold promise for therapeutic intervention in the setting of inflammation and fibrosis in several disease settings, including fibroproliferative lung disease.

Conclusions

There is compelling evidence that the TF-dependent extrinsic coagulation pathway is locally activated in the lungs of patients with ALI/ARDS and fibrotic lung disease and further that PAR_1, the high-affinity thrombin signalling receptor, plays a central role in orchestrating the interplay between coagulation, inflammation and fibroproliferation in experimental models of lung injury via its ability to release and activate a host of pro-inflammatory and pro-fibrotic mediators (Figure 4). Although these responses might be part of the normal programme leading to tissue repair, excessive procoagulant signalling in response to lung injury is highly deleterious. Therapeutic agents aimed at blunting excessive procoagulant activity and signalling may therefore offer promise as novel therapeutic agents in these disease settings. Targeting the lung epithelium directly with novel inhaled therapies (for example, TFPI, APC) may offer advantage over traditional anticoagulant therapy in terms of avoidance of potential bleeding complications. Moreover, therapeutic strategies aimed at targeting PAR_1 directly may offer similar benefits. PAR_1 antagonists are currently being developed as antithrombotic agents and such agents may hold promise in the setting of a number of conditions associated with excessive procoagulant signalling.

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Conflict of interest

The authors state no conflict of interest.

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