Coagulation cascade proteases and tissue fibrosis
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Abstract
Fibrotic disorders of the liver, kidney and lung are associated with excessive deposition of extracellular matrix proteins and ongoing coagulation-cascade activity. In addition to their critical roles in blood coagulation, thrombin and the immediate upstream coagulation proteases, Factors Xa and VIIa, influence numerous cellular responses that may play critical roles in subsequent inflammatory and tissue repair processes in vascular and extra-vascular compartments. The cellular effects of these proteases are mediated via proteolytic activation of a novel family of cell-surface receptors, the protease-activated receptors (PAR-1, -2, -3 and -4). Although thrombin is capable of activating PAR-1, -3 and -4, there is accumulating in vitro evidence that the profibrotic effects of thrombin are predominantly mediated via PAR-1. Factor Xa is capable of activating PAR-1 and PAR-2, but its mitogenic effects for fibroblasts are similarly mediated via PAR-1. These proteases do not exert their profibrotic effects directly, but act via the induction of potent fibrogenic mediators, such as platelet-derived growth factor and connective tissue growth factor. In vivo studies using proteolytic inhibitors, PAR-1 antagonists and PAR-1-deficient mice have provided evidence that coagulation proteases play a key role in tissue inflammation and in a number of vascular pathologies associated with hyperproliferation of smooth muscle cells. More recently, coagulation proteases have also been shown to play a role in the pathogenesis of fibrosis but the relative contribution of their cellular versus their procoagulant effects awaits urgent evaluation in vivo. These studies will be informative in determining the potential application of PAR-1 antagonists as antifibrotic agents.

Introduction
The main function of the coagulation cascade is to ensure the formation of stable haemostatic clots, consisting of aggregated platelets enmeshed in fibrin, that plug injured vessels and prevent blood loss. It has been known for a long time that fibrin deposition plays a pivotal role in influencing subsequent tissue repair processes by acting as a provisional structural matrix for fibroblasts and inflammatory cells migrating into the area of injury and by acting as a reservoir of growth factors and fibrogenic cytokines [1]. The last decade has also seen a major re-evaluation of our perception that

Key words: connective tissue growth factor, Factor Xa, protease-activated receptor, thrombin.

Abbreviations used: CTGF, connective tissue growth factor; EPR-I, effector-cell protease receptor-1; IL, interleukin; PAR, protease-activated receptor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

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the functional roles of coagulation proteases are restricted to blood coagulation. Thrombin and several upstream proteases of the extrinsic coagulation cascade are capable of activating a novel family of cell-surface receptors, the protease-activated receptors (PARs), and thereby exert numerous cellular effects that are likely to play critical roles in subsequent inflammatory and tissue repair processes as part of the normal response to tissue injury (Figure 1). An overexuberant tissue repair response can often lead to the development of tissue fibrosis and loss of function. This can affect a number of organs, but is most commonly observed in the skin, liver, lungs, kidneys and heart. The development of tissue fibrosis is characterized by tissue inflammation, a profound fibroproliferative response and excessive and disordered deposition of matrix proteins. Fibrin persistence has been linked to the development of tissue fibrosis in a number of organs, although recent studies in transgenic animals have given contradictory results [2,3]. This brief review concentrates primarily on evidence supporting a role for PAR-mediated cellular effects in tissue repair responses and the development of tissue fibrosis, but readers are referred to a detailed review [4] for a wider description of the functional roles of PARs and their protease agonists.

**Activation of coagulation proteases**

Activation of coagulation proteases is one of the earliest events following tissue injury involving damage to blood vessels. It is usually initiated when tissue factor, expressed on the surface of vascular smooth muscle cells, activated endothelial cells and platelets following damage to blood vessels, triggers the extrinsic coagulation cascade by binding to circulating Factor VII. However, it is also increasingly recognized that extravascular activation of coagulation proteases and fibrin deposition can occur in certain pathological situations that are associated with increased vascular permeability, inflammation and fibrosis [5–7]. A number of non-vascular cells, including macrophages, fibroblasts and epithelial cells, express tissue factor [8] and are therefore capable of triggering the extrinsic coagulation cascade. Inflammatory cells such as monocytes and neutrophils also express a newly described integral cell surface receptor for Factor Xa, termed effector-cell protease receptor-1 (EPR-1) [9], and therefore support the formation of the prothrombinase complex, responsible for the conversion of prothrombin to thrombin. In addition, certain viruses and bacterial proteases have been reported to promote the generation of Factor Xa [10] and therefore represent another potentially important trigger for the activation of coagulation proteases in the absence of blood coagulation.

**PARs: signalling receptors for coagulation proteases**

Until recently, research efforts were almost exclusively centred on the cellular effects of throm-
bin, the final enzyme of the coagulation cascade. The mechanism by which thrombin elicits these effects was elucidated with the discovery of a novel cell-surface receptor by Coughlin and co-workers in 1991 [11]. This receptor was initially termed the cloned thrombin receptor, but is now more commonly referred to as PAR-1 to distinguish this receptor from more recently characterized PARs. These receptors belong to the family of seven transmembrane G-protein-coupled receptors but, as their name suggests, they display a unique mode of activation involving proteolytic cleavage and unmasking of a tethered ligand which in turn interacts with the receptor within extracellular loop-2 (Figure 2). The resulting conformational changes in the receptor permit the interaction with heterotrimeric G-proteins, which in turn are responsible for initiating cell signalling. To date, four such receptors (PAR-1, -2, -3 and -4) have been identified with distinct N-terminal cleavage sites, tethered ligand pharmacology and protease agonists (reviewed in [4]). Peptide agonists corresponding to the tethered ligand sequence of PAR-1, -2 and -4 are capable of activating these receptors independently of proteolysis and have been extensively used in order to invoke the role of these receptors in mediating the responses of protease agonists.

Thrombin is capable of activating PAR-1, -3 and -4, but it is now generally accepted that the majority of its cellular effects in humans appear to be mediated via the activation of PAR-1. PAR-1 is widely expressed and is present on most cells, including platelets, endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, monocytes, lymphocytes and certain tumour cell lines (reviewed in [4]). In addition to thrombin, several coagulation proteases immediately upstream of thrombin in the coagulation cascade are now being added to the growing list of proteases which are capable of influencing cell behaviour via the activation of PARs. Factor Xa is capable of activating PAR-1 or PAR-2 depending on the cell type examined [12,13], whereas Factor VIIa has

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**Figure 2**

Activation of PARs

The top panel is a diagrammatic representation of the mechanism by which coagulation proteases activate PARs. Proteolytic cleavage of the N-terminus results in the unmasking of a tethered ligand which in turn interacts with extracellular loop-2 of the receptor and initiates cell signalling via heterotrimeric G-proteins. The bottom panel shows the protease cleavage site within the N-terminus for each PAR and their major protease agonists, including non-coagulation proteases (in parentheses). Residues in bold form the tethered ligand region. Synthetic residues corresponding to these sequences are capable of activating the respective PAR independently of cleavage, apart for PAR-3. TF, tissue factor.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Protease cleavage site</th>
<th>Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>TLDFR \underline{SFLLIR}NP</td>
<td>Thrombin, Factor Xa, TF-VIIa-Xa</td>
</tr>
<tr>
<td>PAR-2</td>
<td>SSKGR\underline{SLGK}V</td>
<td>Factor Xa, TF-VIIa-Xa (Trypsin, Tryptase)</td>
</tr>
<tr>
<td>PAR-3</td>
<td>TLPIK \underline{TFRG}AP</td>
<td>Thrombin</td>
</tr>
<tr>
<td>PAR-4</td>
<td>LPAPR \underline{GYPGQV}</td>
<td>Thrombin (Trypsin, Cathepsin G)</td>
</tr>
</tbody>
</table>
been shown to activate PAR-2, although both of these proteases only act at relatively high concentrations when added exogenously. More recently, transient ternary tissue factor–Factor VIIa–Xa complexes efficiently activate PAR-1 and PAR-2 with much greater efficiency, supporting the concept that the tissue factor–Factor VIIa-initiated coagulation pathway is inseparably linked to PAR activation and cell signalling [14].

At present, the physiological functions of PAR-3 and PAR-4 are not well understood. PAR-3 has a very short C-terminal tail and cannot be activated by peptides corresponding to its putative tethered ligand sequence, so it may not be a signalling receptor. In addition, it has different species-specific physiological functions. In mice, it is thought to act as a thrombin-binding site involved in mediating full thrombin-mediated platelet activation. In humans, although PAR-3 is not present on platelets, it is widely expressed in other tissues and may play a role as a binding receptor. PAR-4 has been shown to be highly expressed at the mRNA level in a number of tissues and has been shown to play a critical role in platelet responses in mice [15] and in regulating vascular tone in mice and humans [16]. Several non-coagulation proteases are capable of activating PARs. There is good evidence, for example, that trypsin and mast cell tryptase are important agonists of PAR-2 (reviewed in [4]). Furthermore, PAR-4 is activated by thrombin at high concentrations, but also by trypsin and the neutrophil granule protease, cathepsin G, and has therefore been proposed to represent a more general serine protease receptor than other PAR family members [17].

**Coagulation proteases play a key role in the renewal and repair of damaged blood vessels**

Many of the cellular effects elicited by thrombin are consistent with a key role for this coagulation protease in the repair of damaged blood vessels. Its interaction with platelets at sites of vascular injury not only ensures the rapid formation of haemostatic plugs, but also the release of a number of fibrogenic mediators, including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), that are involved in influencing early vessel repair responses [14]. There is also good in vitro and in vivo evidence that PAR-1 activation by thrombin promotes vascular permeability via both direct effects on endothelial cells and via the release of 5-hydroxytryptamine (serotonin) from platelets and histamine from mast cells ([18] and references therein). These events, along with the induction of endothelial cell surface adhesion molecules [19] and proinflammatory cytokines [20], facilitate the trafficking of inflammatory cells to sites of vessel damage. Furthermore, this protease also contributes to the renewal of damaged cells within the vessel wall by acting as a potent mitogen for vascular smooth muscle cells via activation of PAR-1 [21]. Thrombin is also mitogenic for endothelial cells at low physiological concentrations [22,23]. More recently, it has been reported that Factor Xa can promote vascular smooth muscle cell proliferation by binding to EPR-1 [24] and activate endothelial cells via EPR-1- and PAR-2-dependent mechanisms. In addition, transient ternary tissue factor–Factor VIIa–Xa complexes are also capable of activating PAR-1 and PAR-2 within the vascular compartment. Although these cellular effects may form part of the normal response to vascular injury, there is also good experimental evidence that they may play an important role in a number of vascular pathologies associated with hyperproliferation of smooth muscle cells, neointima formation and atherosclerosis ([24,25] and references therein).

**Coagulation proteases exert proinflammatory effects outside the vascular compartment**

It is increasingly recognized that thrombin also exerts potent proinflammatory effects, which may play important roles in influencing tissue repair and fibrosis outside the vasculature. For example, thrombin stimulates the release of chemoattractants and proinflammatory cytokines, including the interleukins, IL-1, IL-6 and IL-8, by a number of non-vascular cell types, including fibroblasts, epithelial cells and inflammatory cells [26,27]. Similar proinflammatory effects have also recently been reported for Factor Xa and tissue factor–Factor VIIa complexes (reviewed in [28]). Finally, in vivo support for the importance of PAR-1-mediated inflammatory responses in a disease setting was recently elegantly demonstrated in a mouse model of crescentic glomerulonephritis [29].

**Coagulation proteases influence fibroproliferative responses and connective tissue deposition**

The discovery that thrombin was a potent promoter of DNA synthesis and cellular proliferation...
in chick embryo fibroblasts preceded the cloning of PARs by more than a decade [30]. These findings have now been replicated by a number of laboratories and thrombin has been reported to be a potent mitogen for connective-tissue-producing cells derived from a number of tissues which are prone to developing fibrosis, including the lung [31], liver [32], kidney [33] and skin [34]. Experiments with PAR-1-activating peptides and fibroblasts derived from PAR-1 knockout mice revealed that PAR-1 is the predominant thrombin receptor involved in mediating the mitogenic effects of thrombin for fibroblasts [35]. However, thrombin has also been shown to stimulate fibroblast proliferation through a separate non-proteolytically activated thrombin receptor, but the nature of the receptor(s) involved remain(s) at present unknown [36]. There is also increasing evidence that thrombin does not promote fibroblast proliferation directly, but acts by inducing the expression of autocrine growth factors, including PDGF-AA and PDGF-AB [37,38]. These effects are in accord with the recent characterization of a thrombin response element in the PDGF B-chain gene promoter region [39]. Finally, thrombin has also been reported to up-regulate PDGF \( \alpha \)-receptor expression [37], ensuring that the target cell is capable of responding to the autocrine mediator induced by this coagulation protease. In addition, thrombin is also a chemoattractant for fibroblasts [31] and therefore contributes to expanding fibroblast populations at sites of injury by recruiting fibroblasts from neighbouring environments.

As well as being a potent fibroblast mitogen, thrombin also promotes connective tissue protein production and has been shown to upregulate fibronectin production and release by cultured fibroblasts and epithelial cells [40]. In contrast, this protease exerts both stimulatory and inhibitory effects on proteoglycan protein production by endothelial cells [41], but its effects on fibroblast proteoglycan production have not been specifically assessed to date. More recently, we have shown that thrombin stimulates procollagen production in fibroblasts and smooth muscle cells. These effects were predominantly mediated via PAR-1 and increased procollagen gene expression [42,43]. The stimulatory effects on procollagen mRNA levels were delayed by at least 16 h and cycloheximide-sensitive, indicating again that these profibrotic effects are mediated via a newly synthesized secondary mediator. In subsequent studies, we and others showed that thrombin, Factor VIIa and PAR-1 receptor agonists induce the rapid and dramatic expression of the novel extracellular matrix signalling molecules, connective tissue growth factor (CTGF) and cys61 [44,45]. CTGF is a fibroblast chemoattractant, a mitogen and a promoter of connective tissue production. It is also induced in response to TGF-\( \beta \) and has been proposed to be responsible for mediating several cellular effects elicited by this potent profibrotic mediator [46]. Taken together, these findings raise the possibility that CTGF is a common downstream mediator for several pathways leading to extracellular matrix deposition, so that blocking CTGF may represent a very attractive target gene for antifibrotic therapy for conditions associated with both excessive activation of coagulation proteases and TGF-\( \beta \) production. In addition to influencing connective tissue deposition, there is also in vitro evidence that thrombin may influence remodelling of the nascent matrix and wound contraction. For example, thrombin has been shown to promote the transformation of fibroblasts into contractile smooth muscle \( \alpha \)-actin-positive myofibroblasts [47] and to promote collagen lattice contraction [48]. Thrombin has also been shown to activate latent matrix metalloproteinases and regulate their production [49]. This latter effect is mediated by PAR-1, as are thrombin's effects on fibroblast collagen lattice contraction.

Recent studies that were performed in our laboratory to determine the profibrotic potential of other coagulation proteases revealed that Factor Xa was also a potent mitogen for primary adult fibroblasts derived from a number of organs, including the lung, kidney, heart, skin, and liver [50]. Mechanistic studies revealed that these effects were mediated via binding to EPR-1 and the autocrine release of PDGF, with evidence pointing towards PAR-1 as the signalling receptor involved [51]. In these experiments, Factor VIIa was also found to be mitogenic, albeit at supraphysiological concentrations, but Factor IXa had no effect. These findings raise the possibility that Factor Xa may represent another important stimulus for fibroproliferative responses during both normal tissue repair and fibrosis.

Support for an in vivo role for coagulation proteases in promoting connective tissue synthesis and deposition outside the vascular compartment has come from studies in which thrombin and thrombin receptor agonists have been used to enhance wound healing responses and neovascularization in experimental animals [52]. However,
studies performed with PAR-1-deficient mice failed to support a critical role for this receptor in the setting of skin wound healing responses [53]. Research in our laboratory has largely focused on the role of thrombin in the pathogenesis of pulmonary fibrosis. Dramatic activation of the coagulation cascade is frequently observed in the lungs of patients with, and at risk of developing, pulmonary fibrosis associated with both acute and chronic lung injury [54–56]. In order to determine the contribution of the coagulation cascade to excessive deposition of matrix proteins in pulmonary fibrosis, we recently examined the effect of a direct thrombin inhibitor on lung collagen accumulation in an animal model induced by intratracheal instillation of bleomycin, and were able to show that direct thrombin inhibition attenuated this response ([57] and pp. 211–216 in this issue [58]). Similar findings were reported recently using activated protein C in a mouse model of bleomycin-induced pulmonary fibrosis [59]. We are currently performing experiments in a similar model using PAR-1-deficient mice in order to determine whether the protective effects afforded were due to blocking thrombin’s proinflammatory and profibrotic cellular responses or its procoagulant effects (i.e. deposition of intraand extra-vascular fibrin). These experiments should prove informative in determining the potential usefulness of PAR-1 antagonists as therapeutic agents for fibrotic disorders of the lung and other organs.

Summary and conclusions

There is accumulating evidence that thrombin, as well as immediate upstream coagulation proteases, play important roles in influencing several inflammatory and tissue repair processes in both vascular and extravascular compartments. It is also becoming increasingly clear that these proteases may have an important role in the development of tissue fibrosis in a number of conditions associated with excessive and persistent activation of the coagulation cascade. The importance of the cellular versus the procoagulant effects of coagulation proteases in these disease settings is beginning to emerge. PAR-1 blocking strategies have already proved successful in models of restenosis and neointima formation following vascular injury [60]. The use of these agents in the setting of tissue fibrosis now awaits urgent evaluation since therapeutic strategies based on blocking these receptors would circumvent haemostatic complications which often preclude the use of direct inhibitors of coagulation proteases. As we continue to learn more about the way coagulation proteases exert their profibrotic effects, it is also becoming apparent that these effects are mediated via the induction of potent fibroblast mitogens and growth factors. The finding that these secondary mediators may represent a point of convergence for several pathways leading to excessive deposition of matrix proteins may have important implications, in that strategies aimed at blocking these key mediators may represent an even more attractive therapeutic strategy for the prevention of tissue fibrosis than previously realized.

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