Biochimica et Biophysica Acta 1832 (2013) 1018–1027

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Coagulation and coagulation signalling in fibrosis ☆

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ARTICLE INFO

Article history:
Received 13 October 2012
Received in revised form 20 December 2012
Accepted 22 December 2012
Available online 5 January 2013

Keywords:
Fibrosis
Coagulation
Proteinase-activated receptor
Myofibroblast
Extracellular matrix

ABSTRACT

Following tissue injury, a complex and coordinated wound healing response comprising coagulation, inflammation, fibroproliferation and tissue remodelling has evolved to nullify the impact of the original insult and reinstate the normal physiological function of the affected organ. Tissue fibrosis is thought to result from a dysregulated wound healing response as a result of continual local injury or impaired control mechanisms. Although the initial insult is highly variable for different organs, in most cases, uncontrolled or sustained activation of mesenchymal cells into highly synthetic myofibroblasts leads to the excessive deposition of extracellular matrix proteins and eventually loss of tissue function. Coagulation was originally thought to be an acute and transient response to tissue injury, responsible primarily for promoting haemostasis by initiating the formation of fibrin plugs to enmesh activated platelets within the walls of damaged blood vessels. However, the last 20 years has seen a major re-evaluation of the role of the coagulation cascade following tissue injury and there is now mounting evidence that coagulation plays a critical role in orchestrating subsequent inflammatory and fibroproliferative responses during normal wound healing, as well as in a range of pathological contexts across all major organ systems. This review summarises our current understanding of the role of coagulation and coagulation initiated signalling in the response to tissue injury, as well as the contribution of uncontrolled coagulation to fibrosis of the lung, liver, kidney and heart. This article is part of a Special Issue entitled: Fibrosis: Translation of basic research to human disease.

1. Introduction

Following tissue injury, a complex and coordinated wound healing response comprising coagulation, inflammation, fibroproliferation and tissue remodelling has evolved to nullify the impact of the original insult and reinstate the normal physiological function of the affected organ. Tissue fibrosis is thought to result from a dysregulated wound healing response, through continual local injury or impaired control mechanisms. Although the initial insult is highly variable for different organs, in most cases, uncontrolled or sustained activation of populations of mesenchymal cells leads to the excessive deposition of extracellular matrix proteins and gross tissue distortion and eventually can lead to complete loss of organ function. In the most common and fatal form of lung fibrosis, idiopathic pulmonary fibrosis (IPF), epithelial injury of unknown aetiology is thought to progress through dysregulated epithelial–mesenchymal interactions to a vicious cycle of aberrant tissue repair and injury. This leads to the accumulation of fibroblasts and myofibroblasts within organizing extracellular matrix, which underlie areas of injured and reparative epithelium, causing gross distension and remodelling of alveolar septae, which are described histologically as fibrotic foci [27]. In the liver parenchyma, hepatocyte injury, resulting from a variety of insults ranging from viral infection to aberrant lipid metabolism resulting from high fat diet or prolonged alcohol exposure, leads to inflammation and activation of hepatic stellate cells [51]; while prolonged damage to the bile duct epithelium can induce the activation of peri-portal fibroblasts [31,105]. Similarly, various insults to the kidney, ranging from diabetes to autoimmune disease to drug and chemical toxicity, induce inflammation and subsequent wound healing responses, activating mesangial cells and fibroblasts to differentiate into myofibroblasts and ultimately culminate in the deposition of excessive extracellular matrix and compromised kidney function [73].

Coagulation was originally thought to be an acute and transient response to tissue injury, responsible primarily for initiating the formation of fibrin plugs to enmesh activated platelets within the walls of damaged blood vessels in order to prevent further blood loss. However, the last 20 years has seen a major re-evaluation of the role of the coagulation cascade following tissue injury and there is now mounting evidence that coagulation is critical in influencing subsequent inflammatory and fibro-proliferative responses during normal wound healing, in a range of pathological contexts across all major organ systems [21].

This review will summarise our current understanding of the role of coagulation and coagulation initiated signalling in the tissue response to injury and fibrosis. We will introduce the critical events and major components involved in the initiation of coagulation, coagulation signalling

☆ This article is part of a Special Issue entitled: Fibrosis: Translation of basic research to human disease.

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http://dx.doi.org/10.1016/j.bbadis.2012.12.013

and fibrinolysis and then review the evidence for their involvement in fibrosis of the lung, liver, kidney and heart.

2. Overview of the coagulation cascade and fibrinolysis

Extrinsic coagulation is initiated immediately upon tissue injury. Extravascular tissue factor (TF) normally expressed in tissues and concealed from plasma becomes exposed, initiating the formation of the TF–factor VIIa (FVIIa) complex by binding small amounts of circulating FVIIa. This then catalyses the activation of factor X (FX) to FXa. The TF–FVIIa–FXa complex in association with activated factor V (FVa), catalyses the conversion of pro-thrombin to thrombin, which in turn converts fibrinogen to fibrin, initiating the formation of a clot. Explosive amplification of coagulation is subsequently achieved through a feed-forward mechanism via the intrinsic coagulation pathway. Here thrombin catalyses the activation of factors XI, IX, VII and X.

Tight control of coagulation is required, and this is achieved via negative feedback mechanisms involving several locally produced anticoagulants. The extrinsic pathway is principally regulated by TF pathway inhibitor (TFPI), which inactivates the TF–FVIIa–FXa complex. Thrombin, the initiator of the intrinsic pathway, is controlled by a variety of anticoagulants including anti-thrombin, which inhibits thrombin – in addition to other coagulation proteinases – in the presence of heparin, heparin cofactor II and protease nexin-1. α2-Macroglobulin and α1-antitrypsin inhibit thrombin and factors IXa, Xa, and XIa. Protein Z-dependent proteinase inhibitor inhibits FXa in the presence of protein Z, pro-coagulant phospholipids, and calcium. Finally, by binding to thrombomodulin on the endothelium, thrombin is converted from a pro-coagulant into an anticoagulant and activates protein C (PC) bound to the endothelial cell protein C receptor (EPCR). Activated protein C (APC), in conjunction with protein S, inactivates factors Va and VIIIa and thereby suppresses further thrombin generation.

Thrombin mediated cleavage of fibrinogen to fibrin is essential for haemostasis. The provisional matrix of cross-linked fibrin generated by this process is critical to re-establishing blood vessel integrity following injury. Moreover during wound repair, the fibrin matrix acts as a reservoir of growth factors and pro inflammatory cytokines, promoting leukocyte migration, and the accumulation, activation and proliferation of mesenchymal cells. During normal wound healing, the clearance of fibrin via the fibrinolytic system mitigates the risks associated with excessive fibrin accumulation. Fibrinolysis is initiated by the conversion of plasminogen to plasmin by the plasminogen activators urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). The activity of these serine proteinases is regulated by plasminogen activator inhibitor-1 (PAI-1), the expression of which is tightly regulated by a variety of growth factors, including TGFβ, IL-1β and EGF [44].

3. Coagulation signalling: the proteinase activated receptors

Beyond haemostasis, coagulation plays a central role in influencing the subsequent cellular events of inflammation and tissue repair. The existence of thrombin mediated receptor signalling was first hypothesised over 20 years ago, following the observation that thrombin was able to mediate vascular and platelet responses independent of fibrin deposition [142]. Over the intervening years, four G-protein coupled receptors, the proteinase activated receptors (PAR-1 to -4), have been identified which convert extracellular pro-coagulant activity into intracellular signalling events (reviewed in [109]). Though it is now apparent that PARs are expressed on a variety of cells in all organs throughout the body, the role of PARs has been most extensively studied in the vasculature where these receptors are now known to play key roles in influencing platelet aggregation and secretion; vascular contraction and permeability; leukocyte adhesion and the release of nitric oxide [109]. In humans, current evidence suggests a major role for PAR-1 and PAR-2 in mediating signalling in the endothelium, whereas PAR-1 and PAR-4 are central to promoting platelet responses.

Each member of the PAR family is activated by proteolytic cleavage of the extracellular amino terminus, resulting in the cryptic unmasking of a high-affinity tethered ligand which in turn interacts with the ligand binding pocket of the receptor to initiate downstream signalling. Thrombin is a major activator of PAR-1, PAR-3, and PAR-4, but thrombin is also capable of trans-activating PAR-2. Trans-activation occurs following N-terminal cleavage of the PAR-1 tethered ligand, which is then free to interact with the binding pocket of an adjacent PAR-2 receptor [94]. The importance of this transactivation process has recently been highlighted in a mouse model of sepsis. In this model, PAR-1 was found to switch from playing a vascular-disruptive role to playing a vascular-protective role via interaction with PAR-2 during the progression of sepsis [62]. Whereas thrombin interacts directly with PAR-1, coagulation FXa-receptor interactions with the PARs are more complex and the ability of FXa, either on its own, or as part of the more potent TF–FVIIa–FXa ternary complex to activate either PAR-1 or PAR-2 is X dependent on both cell type and cofactor expression [21].

Once activated, PARs couple to multiple heterotrimeric G-protein subtypes including, in the case of PAR-1 and PAR-2, G_i, G_q and $G_{12/13}$. PAR-4 couples G_q and $G_{12/13}$ whereas the signalling properties of PAR-3 remain unclear [23,123]. In keeping with other GPGR systems, the particular G-proteins recruited following PAR activation dictate specific downstream responses. This phenomenon of functional selectivity is exemplified by PAR-1, where at relatively high concentrations, thrombin-induced vascular barrier permeability is mediated by coupling to $G_{12/13}$ and Rho kinase signalling. In contrast, thrombin induced calcium mobilization is mediated by G_q recruitment. Moreover, activation of PAR-1 by synthetic hexapeptide agonists which mimic the tethered ligand appear to induce signalling via G_q, whereas thrombin triggered PAR-1 signalling is preferentially coupled to $G_{12/13}$ [83]. Thrombin concentration also dictates subsequent cellular responses following PAR-1 ligation. This is best exemplified in the endothelium where low concentrations of thrombin (<40 pM) are barrier protective and high concentrations are highly disruptive [35]. Similarly, activation of PAR-1 by the anticoagulant activated protein C (APC) is also barrier protective by mediating the cross-activation of the sphingosine 1-phosphate receptor (S1P1) [36].

4. Coagulation and coagulation signalling in internal organ fibrosis

As alluded to previously, coagulation, which is essential in the early stages of the tissue injury response by promoting haemostasis, is now known to influence several key inflammatory and fibroproliferative responses. Uncontrolled or over-exuberant coagulation therefore retains the potential to induce dysregulation of these tightly regulated processes leading to inappropriate tissue repair and the development of fibrosis (Fig. 1).

4.1. Coagulation and lung fibrosis

Of all the fibrotic conditions, the mechanistic link between coagulation and fibrosis is perhaps strongest for fibroproliferative diseases of the lung in the context of acute lung injury (ALI/ARDS) and in chronic fibrotic conditions including idiopathic pulmonary fibrosis (IPF) and pulmonary fibrosis associated with systemic sclerosis. TF expression is increased by the alveolar epithelium of patients with ALI/ARDS [7] and levels of TF-VIIa are increased in the bronchoalveolar lavage fluid (BALF) of these patients. Critically, increased levels of TF are not matched by a similar increase in the levels of endogenous anti-coagulants such as anti-thrombin III and tissue factor pathway inhibitor (TFPI) [8]. Moreover, recent studies have suggested that microparticles derived from injured alveolar epithelial cells represent an

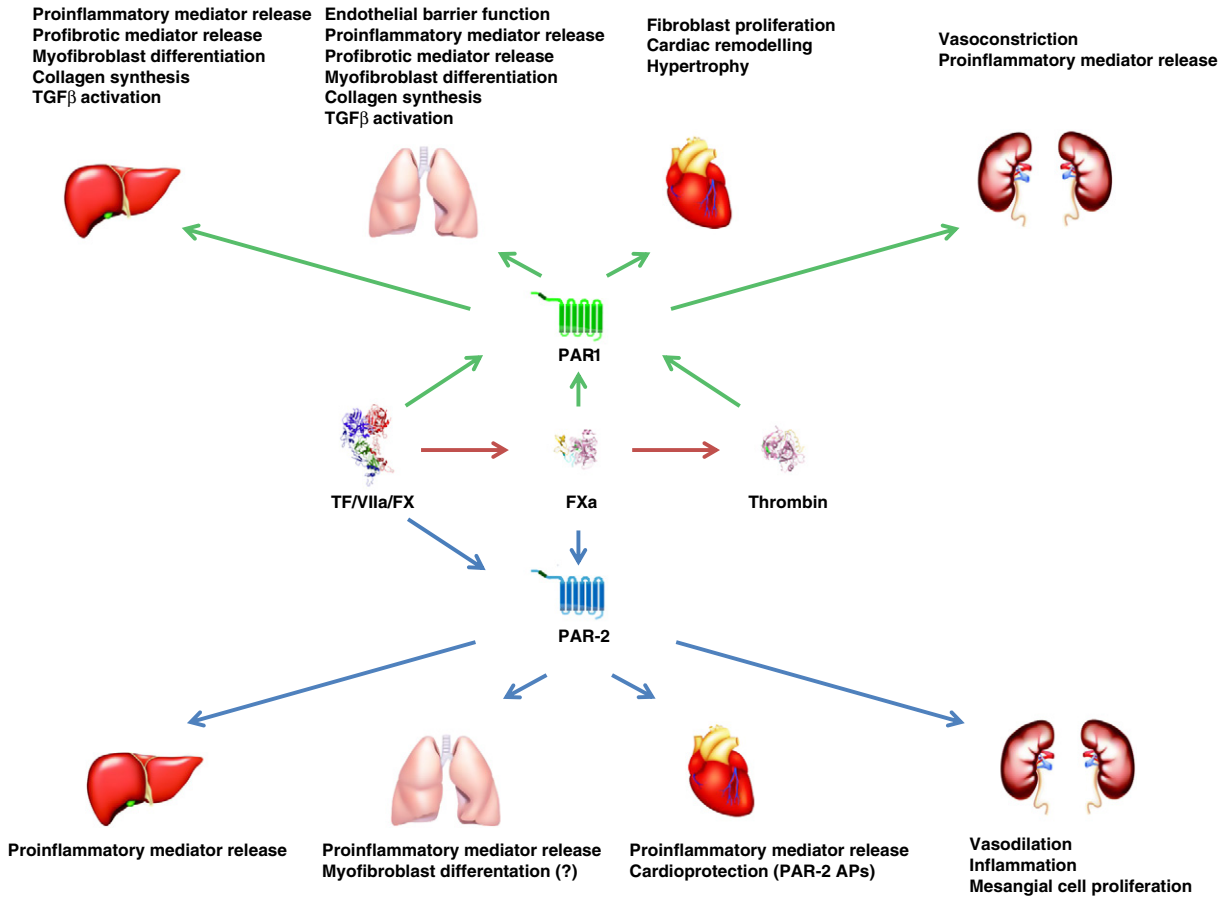

Fig. 1. Proteinase receptor signalling in organ fibrosis. Evidence from in vitro and in vivo studies places PAR-1 as a critical signal transducer for the extrinsic coagulation cascade, influencing processes from endothelial barrier function, inflammation and expression of pro-fibrotic mediators in fibrotic disease in the liver, lung, heart and kidney. The role for PAR-2 is less well defined, however evidence from a variety of studies underlines the importance of this receptor in modulating the inflammatory response.

important source of extravascular pro-coagulant activity in the lungs of patients with ALI/ARDS [6,42].

Likewise in idiopathic pulmonary fibrosis and systemic sclerosis, coagulation is thought to be initiated in the intra-alveolar compartment as a result of increased TF expression by type II alveolar epithelial cells and alveolar macrophages [52,58]. More recently, we have demonstrated that the bronchial and pulmonary alveolar epithelium represent important local cellular sources of several coagulation factors and a nidus for FX activation in patients with IPF and in the bleomycin model of lung injury and fibrosis [117].

In terms of pro-coagulant signalling responses in the injured lung, current evidence suggests a key role for PAR-1 in influencing a range of cellular responses with the receptor being highly expressed on the endothelium and on the injured epithelium and fibroblasts within fibrotic foci [84]. Activation of PAR-1 induces the synthesis and release of a range of pro-inflammatory mediators, including chemokines such as CXCL8 and CCL2 and cytokines such as TNFα, IL-1β, IL-2 and IL-6 [28,74,97,117]. Moreover thrombin has been reported to induce the expression of a variety of adhesion molecules on endothelial cells [107]. Activation of PAR-1 on human lung fibroblasts has been shown to induce synthesis of the potent anti-fibrotic eicosanoid PGE2, which in turn negatively regulates the expression of PAR-1. COX-2 mediated PGE2 synthesis is disrupted in fibrosis [65], and fibrotic fibroblasts show a diminished capacity to synthesise PGE2 in response to thrombin stimulation [124]. These observations may explain why PAR-1 is over expressed in the lungs of patients with IPF [84]. Early in vitro studies on fibroblasts demonstrated that activation of PAR-1 with either thrombin or FXa promotes their proliferation via the autocrine induction of PDGF [13]. Activation of this receptor also induces pro-collagen production by fibroblasts [19], and induces synthesis of connective tissue growth factor (CTGF) [20], fibronectin [5] and plasminogen activator inhibitor (PAI-1) [50]. Moreover, thrombin treatment of lung fibroblasts induces their differentiation into myofibroblasts [14].

In terms of evidence for a role for coagulation signalling in driving fibrosis in animal models, we and others have shown that anticoagulant strategies (e.g. direct thrombin inhibition) and PAR-1 deficiency are protective in the bleomycin model of lung injury and fibrosis, with protection associated with reduced levels of several profibrotic mediators, including CTGF and TGFβ and the pro-inflammatory and pro-fibrotic chemokine CCL2/MCP-1/JE [55]. More recently, PAR-1 has also been shown to play a key role in mediating the integrin-dependent activation of latent TGFβ, one of the most potent pro-fibrotic mediators characterized to date. In epithelial cells, this response was found to be mediated via the activation of the epithelial restricted αvβ6 integrin, which is currently also a major drug target for anti-fibrotic therapy [61]. In contrast, we and others have shown that fibroblasts/myofibroblasts do not express αvβ6 but express high levels of αvβ5 within fibrotic foci and ligation of PAR-1 similarly leads to αvβ5 integrin dependent TGFβ activation [117].

In contrast to PAR-1, the role of PAR-2 in lung injury and fibrosis seems less clear. Critically there is differential expression of PAR-2 in human and murine lungs, potentially confounding correlations between murine models and disease. While PAR-2 is observed on the pulmonary epithelium in both mice and humans, and has been shown to mediate proliferation of primary human lung fibroblasts in vitro, the receptor is not generally thought to be expressed by mouse lung fibroblasts [150]. This is not a universal finding however.

One study suggests that PAR-2 is expressed on myofibroblasts in the murine lung, modulating tissue factor expression and driving myofibroblast differentiation following bleomycin injury [15], while others have found that deficiency in PAR-2 offers no protection [129]. In other murine models of lung injury direct activation of PAR-2 using specific hexapeptides induces increased airway tone, acute lung inflammation, and extravascular leak via a neurogenic mechanism however PAR-2 does not seem to be critical in models of *Escherichia coli* induced pneumonia [8,128].

4.2. Coagulation and liver fibrosis

In comparison to IPF where the aetiology of the insult is unknown, the nature of the injurious insults leading to hepatocyte damage and liver fibrosis are much better defined and include hepatitis B or hepatitis C viral (HBV or HCV) infection or aberrant lipid metabolism induced by prolonged alcohol exposure or obesity. In chronic cholangiopathies, bile duct epithelial cells are the primary site of injury, resulting from either auto-immune mediated cellular damage, or build-up of bile resulting from bile duct blockage. Injury to either hepatocytes or BDECs results in the local upregulation of inflammation and activation of local resident mesenchymal cell populations, the hepatic stellate cells (HSCs) and peri-portal fibroblasts respectively. Once activated both cell types differentiate into contractile myofibroblasts, capable of maintaining the expression of pro-inflammatory and pro-fibrotic mediators, and the deposition of extracellular matrix.

Hepatocytes are critically important as the major cellular source of vitamin K required for the gamma-carboxylation of vitamin K dependent coagulation factors including factors II, V, VII, IX and XI. Hepatocyte loss due to injury, for example in severe cirrhosis, results in a systemic deficit in these factors (reviewed in [16,138]) causing the hypo-coagulation commonly described in liver disease. Additionally there is also loss of critical vitamin K dependent anticoagulants, including protein C and protein S [98,106]. In order to mitigate these effects, patients with liver disease and cirrhosis are often prescribed vitamin K [114]. Intriguingly, it has recently been argued that, rather than systemic hypo-coagulation, the concurrent loss of pro and anti-coagulant factors resulting from liver injury actually results in a net rebalancing of the coagulation cascade, offering the potential for a hyper-coagulant state associated with liver disease [139].

Additional factors contributing to an enhanced pro-coagulant state in the injured liver include decreased blood flow, endothelial dysfunction or chronic inflammation [17,154]. Underlining the involvement of coagulation in liver injury and fibrotic progression, numerous studies have observed microthrombus formation in the hepatic vasculature in both rodent models of injury [70,71,76,77] and in cirrhotic patients [9,144,145]. The functional role of increased coagulation activity in the liver was highlighted in studies showing that carriage of the factor V Leiden (FvL) mutation, a common cause of hereditary hypercoagulopathy, was associated with increased risk of rapid fibrosis progression following hepatitis C (HCV) infection [106,149] in humans. Moreover, mice carrying this mutation exhibited a more severe fibrosis than littermate controls [2], a finding which echoes observations reported in the bleomycin model of lung injury and fibrosis [152]. These observations lead to the proposition of the parenchymal extinction hypothesis of liver injury, whereby loss of blood flow induced by hypercoagulation and thrombus formation, propagates hepatocyte apoptosis, tissue damage, and ensuing fibrosis [144,145]. However, evidence also exists for an alternative hypothesis involving coagulation signalling actively driving a pro-fibrotic programme in the injured liver.

In support of this notion a recent genetic study based on Brazilian and European patient cohorts, has highlighted that a polymorphism in the 5′ regulatory domain of PAR-1 (1426 C/T) shows a strong association with the rate of fibrosis induced by HCV infection [81]. The cellular localisation of PARs has also been assessed in the fibrotic liver, where PAR-1 (referred to as the thrombin receptor), was found to localise to hepatic stellate cells [80]. Subsequent studies in the rat, have identified the expression of PAR-1, PAR-2 [38,41], and PAR-4 [38] on HSCs. PAR-1 activation on HSCs was found to promote myofibroblast differentiation in vitro, with receptor expression increasing during the differentiation programme [38,41].

Functional studies in mice have also provided evidence for a link between coagulation signalling and the development of non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of obesity. Mice fed a Western chow diet and expressing low levels of haematopoetically expressed TF or PAR-1 deficiency demonstrated reduced inflammation (CCL2 expression and macrophage accumulation), and reduced steatosis (hepatocyte lipid accumulation), compared to wild type controls [63]. In a similar model, the direct thrombin inhibitor Argatroban, dosed therapeutically (i.e. post development of steatosis), reduced the expression of several inflammatory and fibro-proliferative biomarkers in the liver, including TGFβ, TIMP-1, collagen and αSMA [64].

Cholangiopathies in humans are also associated with increased expression of TF by bile duct epithelial cells (BDECs), as well as with increased thrombin generation and expression of PAR-1. These observations are mirrored in rodent models of bile duct epithelial injury, induced by the BDEC selective toxicant α-napthylisothiocyanate (ANIT), and bile duct ligation [12,119,130]. In the ANIT model of BDEC injury, deficiency in either TF or PAR-1 is associated with reduced expression of several pro-fibrotic genes and reduced pro-fibrotic signalling responses [75,130], while either anticoagulation or PAR-1 antagonism attenuates subsequent collagen accumulation in the bile duct ligation model [1,38]. Intriguingly, reduced TF or PAR-1 signalling was associated with the impaired ability of BDECs to induce αvβ6 gene expression in response to TGFβ stimulation [130]. As mentioned earlier, this integrin has been previously shown to be critical in mediating TGFβ activation in pulmonary epithelial cells following PAR-1 induced cell contraction [61]. Collectively these data point to an important role for coagulation in driving an over-exuberant response to injury, and subsequent fibrosis in the liver.

4.3. Coagulation and renal fibrosis

Glomerulosclerosis and tubulointerstitial fibrosis represent the fibrotic endpoint of a range of chronic insults to the kidney, grouped together under the pathology of chronic kidney disease (CKD). Coagulation has also been shown to be activated in CKD, with mounting evidence suggesting imbalances in both the coagulation cascade and the subsequent fibrinolytic system (reviewed in [59]). Plasma TF levels, as well as FXIIa and FVIIa activity, have been reported to be increased in patients with CKD [82,99]. Moreover, activated protein C complex [132] and thrombin–anti-thrombin complex levels have been shown to be increased whereas anti-thrombin activity is reduced [135]. In an animal model of hydronephrosis, TF expression was shown to be increased in vascular endothelial cells, vessel wall, tubular epithelial cells, and glomerular capsular cells [147].

Despite the observations implicating coagulation signalling in CKD, a mechanistic link between the coagulation cascade to and underlying pathology remains to be clearly demonstrated. Studies in mice and humans support the notion that coagulation signalling, via proteinase activated receptors, may play a role both in maintaining kidney function, and in controlling the early events of kidney injury leading to fibrosis. PAR-1 and PAR-2 expression has been reported in the kidney, with both receptors co-localising to renal vascular and tubular interstitial cells [11,26,46,111]. Intriguingly, activation of either PAR-1 or PAR-2 elicits divergent haemodynamic consequences. In an isolated perfused model of kidney function, PAR-1 activation was reported to initiate renal vasoconstriction and a marked reduction in the glomerular filtration rate (GFR), whereas PAR-2 activation contrastingly caused vasodilation and partial reversal of the PAR-1 mediated effects [47].

It has long been recognized that ischaemia is also a major cause of renal injury leading to fibrosis [85]. A role for PAR-1 in ischaemia

reperfusion (I/R) injury has been suggested in various organ settings including the intestine and the heart [126,140]. In the kidney, studies using either low TF expressing or PAR-1 −/− mice suggest that increased TF activity after renal IR leads to increased CXC chemokine expression and subsequent neutrophil-mediated injury via a PAR-1 dependent mechanism [120]. In agreement with these findings, more recent studies have also reported that rats treated with the PAR-1 antagonist, SCH7979, also exhibit reduced inflammation in a model of ischaemia reperfusion [33]. Both studies suggest that, in addition to regulating kidney haemodynamics, activation of PAR-1 leads to the up-regulation of several pro-inflammatory mediators including TNFα, KC, MIP-2 and CINC-1, in addition to increasing the expression of adhesion molecules such as P-selectin [33,120].

PAR-1 deficiency was also shown to be protective in a model of glomerulonephritis, with PAR1 −/− mice showing less severe renal failure, macrophage infiltration and fibrin deposition than wild type controls [25]. Infusion of a PAR-1 specific hexapeptide agonist into wild type animals exacerbated the injury. Additionally a potential role for factor Xa mediated ligation of PAR-2 has also been highlighted by in vitro studies showing that factor Xa can induce mesangial cell proliferation through PAR-2 mediated ERK activation [134].

Anticoagulants have proven efficacious in renal models of fibrosis, suggesting a role for the coagulation cascade in mediating pathology, however findings need to be interpreted with caution. Studies using either low molecular weight heparin or the FXa inhibitor, fondaparinux, reduced inflammation in renal ischaemia reperfusion [40], urethral obstruction [103], and in diabetic nephropathy models [131], with the latter studies also showing attenuation of fibrotic markers in addition to inflammation. However, both heparin [18] and fondaparinux [39] exert anti-inflammatory effects which are independent of their anticoagulant activities, so it is possible that protective effects were mediated via the non-anticoagulant effects of these agents. Despite this caveat, the fact that they have proved efficacious in renal injury models certainly strengthens the case for their use as renal anti-fibrotics in CKD.

4.4. Coagulation and cardiac fibrosis

Similar to other organs, injury to the myocardium induces a wound healing response with the potential, if inappropriately controlled, to develop into fibrosis. Despite occupying 75% of the tissue mass of the heart, cardiac myocytes only account for 30% of the cell population, with the remaining portion being made up of interstitial fibroblasts [22]. Excessive extracellular matrix (ECM) deposition by activated fibroblasts increases the stiffness of the myocardium; moreover, aberrantly deposited ECM impairs the mechano-electric coupling of cardiomyocytes increasing the risk of arrhythmias (reviewed in [68]). Collectively these pathologies lead to the functional impairment of the heart with a progressive decline to cardiac failure.

The role of the coagulation cascade in cardiac fibrosis as in other organs is highly complex. While complete deficiencies in TF, FVII, FX, FV, and pro-thrombin are fatal in utero or shortly after birth, mice expressing low-TF (≈1% of wild type levels) survive, but are prone to spontaneous haemorrhage in organs such as the heart, lung, brain, uterus and placenta [53,100,112]. In contrast to other organ settings, deficiency in TF spontaneously induces cardiac fibrosis which is evident in these animals from as early as three months after birth. TF deficiency seems to influence fibrosis in a dose-dependent manner, with ECM deposition completely reverted following rescue by TF overexpression [100,102]. Critically, the myocardium of low-TF expressing mice shows evidence of haemorrhage associated with areas of fibrosis, suggesting that this could be the trigger for the fibrotic response in these animals. It has been hypothesised that, due to the nature of the continual mechanical stress to which it is subject, the myocardium is prone to repetitive mechanical injury. High TF expression may act as a secondary haemostatic barrier against intra-myocardial haemorrhage, which is diminished when TF expression is compromised.

In contrast, in the context of an acute injury to the heart, excessive activation of coagulation appears to be deleterious in line with other organ systems. Myocardial infarction (MI) and subsequent breach of the endothelial barrier allows leakage of coagulation factors into the myocardium and subsequent activation by myocardial TF [34]. Moreover, in rabbit models of cardiac ischaemia reperfusion (I/R), inhibition of TF or thrombin has been shown to be protective [45]. With regard to coagulation signalling, PAR-1 is expressed in the heart by cardiomyocytes, fibroblasts, smooth muscle cells (SMCs) and endothelial cells [3], with expression increased in patients with various forms of heart failure [4,88]. A recent study suggests that PAR-1 is the most highly expressed GPCR expressed on cardiac fibroblasts isolated from rat hearts [122]. While it is still unclear from genetic and pharmacological studies as to whether PAR-1 is involved in the insult of the myocardial infarct [101,126], evidence suggests a role in the wound healing response. PAR-1 −/− mice exhibit reduced hypertrophy post-MI, while transgenic animals engineered to overexpress PAR-1, specifically on cardiomyocytes, showed evidence of cardiac remodelling and hypertrophy [101]. Moreover, PAR-1 signalling in cardiac fibroblasts induces their proliferation [57,125].

PAR-2 expression is increased in the hearts of patients with ischaemic heart failure and mice following cardiac I/R injury. Moreover, mice deficient in PAR-2 exhibit reduced cardiac I/R injury, associated with reduced oxidative stress and inflammation [4]. These data contrast with other studies where PAR-2 activating peptides in vivo and in isolated perfused hearts, were shown to be protective in cardiac I/R injury [90,91]. In accounting for these contradictory data, it has been proposed that peptide mimetics induce 'biased agonism' at the PAR-2 receptor, where differential signalling is induced by different activators and peptide agonists [108]. It has been proposed that PAR-2 activating peptides elicit a change in the 3-dimensional structure of PAR-2 distinct from that induced by proteolytic activation, initiating the recruitment of differential G-protein or β-arrestin mediated signalling pathways [3].

Genetic and pharmacological investigations of the role of PAR-4 in cardiac injury have also yielded divergent data. Studies using PAR-4 −/− mice suggest that this receptor is cardio protective in I/R injury [3], however pharmacological studies with the pepducin antagonist of PAR-4, P4pal 10, and the trans-cinnamyol-YPGKF-amide have suggested that antagonism of this receptor is protective in I/R injury [127]. The interpretation of data using these antagonists is potentially confounded however by limitations of the bioavailability of these agents in vivo as well as the observation that both behave as agonists in some in vitro model systems [54]. Collectively, these observations suggest a significant role for coagulation receptor signalling in both the initiation of cardiac injury as well as the subsequent development of fibrotic responses.

4.5. The role of fibrinolysis in organ fibrosis

Numerous clinical studies have linked elevated levels of PAI-1, the major serpin regulator of fibrinolysis, to fibrosis in each of the major organ settings. PAI-1 levels are elevated in the lungs of patients with ALI and IPF [66,116,146]. In the cirrhotic liver, levels are down-regulated in a subset of patients [56], however conversely synthesis of PAI-1 is induced by alcohol administration [30,79,89] with increased levels acting as a measure of disease severity in the early stages of liver disease [136]. In renal fibrosis, fibrin deposition in the peritubular capillaries and the interstitial space is common and numerous studies have found that PAI-1 is elevated during the progression of kidney fibrosis (extensively reviewed in [113]). In the main, these clinical findings have been mirrored in animal models which have shown that either PAI-1 deficiency or supplementation of u-PA (both resulting in enhanced fibrinolysis) is associated with attenuated fibrosis following bleomycin-induced lung injury and fibrosis [32,48] and renal fibrosis [67,96] and models of liver fibrosis which

mimic the effects of alcohol induced steatosis [10] or bile duct obstruction [143]. However beyond these, review of the literature investigating the contribution of the components of fibrinolysis to organ fibrosis yields conflicting outcomes which appear dependent on the animal model used. For example PAI-1 deficiency is associated with exacerbation of liver fibrosis in a severe carbon tetrachloride model targeting hepatocytes [141], while paradoxically overexpression of u-PA is protective in this model [92,115]. It was noted that hepatocytes in PAI-1 −/− mice were unable to proliferate and replenish those lost to injury, leading to the suggestion that this functional deficit exacerbated the fibrotic effects of a severe hepatocyte targeted injury [141]. Moreover, PAI-1 deficiency was shown to be either protective or deleterious depending on the model of glomerular nephritis employed. In the bleomycin model of lung fibrosis, despite the fact that PAI-1 −/− mice were protected from fibrotic injury, fibrinogen knock-out mice showed no protection suggesting, in this model at least, that fibrin deposition is not required for the development of fibrosis [49].

Strikingly, in the setting of cardiac fibrosis, PAI-1 deficiency promotes spontaneous age-related cardiac fibrosis with studies suggesting that micro-vascular leak in PAI-1 −/− mouse hearts may provoke inflammation, and predispose these mice to cardiac fibrosis [43,86,151]. PAI-1 induces the internalization of αvβ3, with increased expression of this integrin associating with increased TGFβ-dependent SMAD2/3 signalling in fibroblasts derived from PAI-1 deficient mice [104]. It is postulated that increased TGFβ signalling in the myocardium of these animals, in addition to increased inflammation and MMP activity may drive spontaneous cardiac fibrosis. It is striking that the heart is particularly sensitive to deficiency in both coagulation factors (e.g. TF) and PAI-1. As previously argued, the haemostatic balance in the heart is particularly delicately balanced and sensitive to perturbation as a result of the continual mechanical strain it operates under [151]. Paradoxically, following an acute insult in the form of myocardial infarction, PAI-1 deficient mice are protected from fibrosis induced by myocardial infarction [133].

In addition to promoting fibrin clearance, the tPA/uPA/plasmin system is critically involved in the activation of a variety of MMPs and growth factors. For example, plasmin is known to directly activate MMP-1, MMP-3, MMP-9, MMP-10 and MMP-13, and is indirectly involved in the activation of MMP-2, which can also be activated by u-PA [60,72]. MMPs play a pleotropic role in the wound healing response, affecting not only matrix turnover and the subsequent release of sequestered growth factors, but also regulating the activity of a host of cytokines and chemokines [110]. MMP-2, MMP-9 and MMP-13 have all been shown to be involved in activation of TGFβ, which is also known to be activated directly by plasmin either by direct cleavage of the latency associated peptide (LAP) factor or indirectly via thrombospondin-1 (TSP-1) expressed on macrophages [60]. The observation of plasminogen activators playing a fibrotic role appears to be at odds with the observation of increased PAI-1 expression in fibrotic disease. However critically the plasminogen activators u-PA and t-PA activate the anti-fibrotic mediator hepatocyte growth factor (HGF), and it has been argued that the ability of the plasminogen activators u-PA and t-PA to activate anti-fibrotic growth factors such as HGF [24,44] is critical for their anti-fibrotic function.

In summary, the fibrinolytic system mediates the dual roles of fibrin clearance and the activation of growth factors involved in tissue repair. However interpreting the role of fibrinolysis in fibrosis is confusing since it is difficult to tease out the relative importance of both roles using current knock out approaches.

5. Therapeutic potential and challenges of blocking coagulation in fibrosis

Current pre-clinical evidence suggests that targeting coagulation signalling could be beneficial in the context of fibrosis of the lung, liver, kidney and possibly heart. However, the development of anticoagulants in the clinical context of fibrosis is challenging on a number of fronts. Aside from the usual challenges to fibrotic drug development, namely proving efficacy in a slowly evolving pathology, the use of anticoagulants in the clinic may carry a significant safety risk. As we have highlighted, coagulation exerts highly complex effects on haemostasis, inflammation and tissue repair so that the beneficial and deleterious effects of anticoagulants are finely balanced. Examination of efforts to target coagulation in lung fibrosis provides a working case study in the difficulties in targeting coagulation in organ fibrosis, with the early promise shown by anticoagulants in animal models and small clinical studies of lung fibrosis proving challenging to translate in large scale clinical trials [69,118]. Recently, a phase III multicentre trial "Anti-Coagulant Effectiveness in Idiopathic Pulmonary Fibrosis (ACE-IPF)", assessing orally dosed warfarin for the treatment of IPF, was terminated early due to futility and increased mortality in the treatment arm [93]. The study findings suggested that excess mortality in the treatment group was attributable to a worsening in respiratory symptoms, rather than being related to side effects of anti-coagulation. The underlying reasons for this unexpected result are not fully understood. The ACE-IPF investigators suggested that causative factors could include alveolar haemorrhage, which was not measured in the study, or the unexpected detrimental effects of inhibiting all vitamin-K dependent coagulation factors (factors II, VII, IX and X) and critical anti-coagulants, such as protein-C and protein-S [93]. The effects on the protein-C axis might be particularly important since activated protein-C (APC) has been shown to exert endothelial barrier protective effects [37]. It is plausible to suggest that loss of the beneficial effects of protein-C could be detrimental in the setting of IPF.

While the ACE-IPF trial strongly argues against systemic depletion of vitamin-K dependent coagulation factors in IPF, the potential for local anticoagulation strategies with agents differentiated from warfarin remains to be explored. Heparin exhibits a different mechanism of action to warfarin, with only partial overlap of coagulation factor targets which are inhibited. Encouraging recent data has emerged from safety and tolerability studies in IPF where patients were administered inhaled heparin, rather than being systemically exposed to the anticoagulant. Patients in the inhaled heparin treatment group showed significant inhibition of local intra-alveolar coagulation without any heparin related side effects [78]. These studies offer the exciting possibility of treatment with a topically targeted anticoagulant in the treatment of IPF.

In contrast to a broad-spectrum anticoagulant therapy, an alternative strategy is potentially offered by selectively targeting the deleterious effects of coagulation signalling responses. To this end a variety of inhibitors of the proteinase activated receptors (PARs), in particular PAR-1 are under development (comprehensively reviewed in [109]). Of all the strategies yet employed for inhibition of PAR-1, small molecule antagonists directed against the tethered ligand binding pocket of the receptor have been the most successful with two antagonists (vorapaxar and atopaxar) entering large scale clinical trials as novel antithrombotic agents in several cardiovascular indications [87,95,137,148]. To the best of our knowledge, there are currently no trials planned for PAR-1 antagonists in the clinical setting of fibrosis, however a recent trial in the setting of acute coronary syndrome has been completed. Disappointingly, the primary end point of the study was not met, while additionally the study was terminated due to increased inter-cranial bleeding in patients with a history of stroke [137]. Vorapaxar is a highly specific and virtually irreversible antagonist at PAR-1, and it may be that an antagonist with a more reversible profile is preferable in this clinical setting [153]. Moreover the findings of this study are potentially confounded by the fact that vorapaxar was administered in addition to standard anti-platelet agents (aspirin and clopidogrel) suggesting that PAR-1 antagonists should not be used in patients in combination with other anti-platelet agents. If these safety considerations were successfully

overcome, it is tempting to speculate that this class of compound may be worth investigating in the clinical settings of lung, liver and kidney fibrosis.

In contrast to the lung, in the liver, no large scale clinical trials of anticoagulants have been completed to date, however, promising findings have been observed in small short duration anti-coagulant studies. Low molecular weight heparin dosed over a three week period was shown to improve levels of serum markers of liver injury and reduce serum type IV collagen and hyaluronic acid in patients with chronic hepatitis B infection compared with patients on normal liver support therapy [121]. In a second study, HCV infected patients who were unresponsive to interferon based antiviral strategies were dosed for 8-weeks with warfarin, resulting in reduced liver stiffness and serum biomarkers of fibrosis [29]. These promising data have triggered a further multicentre trial of warfarin in post-transplant patients infected with HCV [2]. The results of these studies are eagerly awaited.

6. Concluding remarks

Coagulation has evolved to promote haemostasis with a view of rapidly reinstating organ function following injury and as such it is a central mechanism of survival. Over the past 20 years however evidence for a more pleiotropic role for coagulation has emerged. As well as influencing the formation of the provisional matrix, several coagulation proteinases exert key cellular responses to injury via the activation of PARs which show regulated expression in every tissue compartment of the body. As a result, the coagulation cascade directly influences several key aspects of the wound healing response, from platelet aggregation and vasoconstriction to inflammation right through to scar formation. Though tightly regulated under normal conditions, an imbalance in favour of a procoagulant state as occurs in many organ pathologies has the potential to dysregulate inflammatory and tissue repair programmes and culminate in fibrosis. The multifaceted nature of coagulation means that the development of therapeutics in this area is challenging, however the development of compounds which target key cellular responses of coagulation signalling continues to give hope for the development of novel targeted therapies in this area.

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