Thrombin-mediated cellular events in pulmonary fibrosis associated with systemic sclerosis (scleroderma)

A. Ludwicka-Bradley, G. Bogatkevich, R.M. Silver

ABSTRACT
The vascular hypothesis for the pathogenesis of systemic sclerosis was perhaps Professor LeRoy’s most important scientific contribution. One early and important consequence of vascular injury is the release of activated thrombin. In this manuscript we present our data and review the current understanding of the role played by thrombin in the process of fibrosis, particularly as it relates to scleroderma lung disease. Thrombin’s cellular effects are intimately involved in promoting myofibroblast differentiation, endothelial cell activation, extracellular matrix protein deposition, and the induction of important profibrotic factors. Such studies confirm that thrombin is one of the major mediators in the development and progression of pulmonary fibrosis. Therefore, targeting the major receptor of thrombin, PAR-1, and its downstream signaling molecules may lead to novel therapeutic approaches for the management of scleroderma lung fibrosis. We are indebted to Dr. LeRoy for his many contributions to the field of scleroderma, and for all that he did to stimulate our interest in these studies.

Introduction
Pulmonary fibrosis in systemic sclerosis (SSc, scleroderma) is an irreversible and progressive disease process often leading to death (1-3). Characterized by microvascular and tissue injury and inflammation, it culminates in excessive deposition of extracellular matrix (ECM) proteins, often resulting in severe lung dysfunction (3-6). Cells predominantly responsible for ECM accumulation in the lung are activated fibroblasts or myofibroblasts (7-13). The presence of myofibroblasts in human and animal models of pulmonary fibrosis is now well documented (14-17). Myofibroblasts may arise from differentiation of fibroblasts or other precursor cells, e.g. pericytes, endothelial cells, epithelial cell and fibrocytes (17-20). Myofibroblasts appear to be the principal mesenchymal cells responsible for tissue remodeling, collagen deposition, and the restrictive nature of the lung parenchyma associated with pulmonary fibrosis (8-10,13,16).

The conceptual process of fibrogenesis involves the presence of tissue injury, the release of fibrogenic factors, and the induction of myofibroblasts, culminating in enhanced extracellular matrix deposition (2, 3, 21-23). Several factors capable of inducing the myofibroblast phenotype have been described. Transforming growth factor-β1 (TGF-β1), a factor that also plays a central role in promoting ECM protein synthesis, is perhaps the best studied. Recently, we have reported that the serine protease, thrombin, also mediates the differentiation of lung fibroblasts to a myofibroblast phenotype, apparently at an even earlier stage than TGF-β1 (27, 28).

In recent years, increasing evidence has accumulated to implicate involvement of the coagulation system in various fibrotic diseases, including pulmonary fibrosis (29-36). Activation of coagulation proteases, e.g. thrombin, is one of the earliest events following tissue injury (29, 33, 36). Activation of the coagulation system and generation of thrombin following injury modulates tissue repair responses by altering vascular permeability, stimulating fibroblast and neutrophil migration, and promoting the adhesion and spreading of endothelial cells, epithelial cells and fibroblasts (33-37). Therefore, during lung injury thrombin activates various cell types and induces the secretion of several profibrotic and angiogenic factors (37-42). Activation of these cells by thrombin is a likely mechanism for the development and progression of pulmonary fibrosis in general, and lung
fibrosis associated with scleroderma in particular.

Role of thrombin in pulmonary fibrosis

There is compelling evidence that the serine protease and G protein coupled receptor ligand, thrombin, is an important mediator of idiopathic pulmonary fibrosis (IPF), interstitial lung fibrosis associated with SSc, chronic asthma, and animal models employing bleomycin to induce lung fibrosis (30-40, 44-48). We, as well as others, have demonstrated elevated levels of thrombin in bronchoalveolar lavage (BAL) fluid from scleroderma patients with lung fibrosis (29-31), as well as for other fibrosing lung diseases (36). We found that BAL fluids from normal subjects contain a low level of thrombin activity (48.6±8.7 U/mg of protein), while BAL fluids from SSc patients contain significantly higher thrombin activity (699.9±201) (29).

Thrombin is a potent mitogen for lung fibroblasts (27-31). Previously, we reported that the mitogenic effect of thrombin on human lung fibroblasts is mediated mainly via PDGF-α receptor up-regulation and enhancement of PDGF-AA ligand expression (29). Recently we demonstrated that thrombin-induced DNA synthesis in human lung fibroblasts is mediated by PKC (48). Thrombin also induces the expression of profibrogenic factors, e.g. transforming growth factor-β (TGF-β1), in smooth muscle cells and epithelial cells (39,47). Moreover, thrombin is a potent stimulator of connective tissue growth factor (CTGF) in fibroblasts (36,49-51). Both of these growth factors are known to participate in various fibrotic diseases, including SSc pulmonary fibrosis (43, 49, 50-55). Levels of each are elevated in BAL fluids and sera from scleroderma patients (43). CTGF mediates some of the profibrotic functions of TGF-β and has been shown to act synergistically with TGF-β to promote chronic fibrosis (54, 55). The presence of the active form of thrombin, together with PAR-1 and CTGF, was recently demonstrated in bleomycin-induced pulmonary fibrosis (49). It has also been demonstrated that CTGF mediates matrix production in lung fibroblasts, which seems to be central to the development of the fibrogenic response, and that inhibition of CTGF activity may be an effective treatment for pulmonary fibrosis (49). Overexpression of CTGF in various fibrotic tissues, including lung, has been observed, with myofibroblasts being the cells mainly responsible for CTGF production (43, 54, 55). High levels of CTGF in lung tissue have been shown to correlate with high collagen synthesis in scleroderma patients (43).

Thrombin has been demonstrated to enhance extracellular matrix proteins, e.g. fibronectin, by epithelial cells and fibroblasts, and procollagen by smooth muscle cells (37, 38, 40) and endothelial cells (41). Studies from our laboratory have shown that thrombin is a potent inducer of tenasin C in human lung fibroblasts (35). We have also shown that thrombin induces interleukin-8 (IL-8) in lung fibroblasts (34). Levels of IL-8 are elevated in BAL fluids of scleroderma patients (56). Thus, thrombin is a potent inducer of profibrotic factors and ECM proteins in various cells within the lung microenvironment. Each of these factors is believed to play an important role in the development and progression of SSc lung fibrosis (29, 34-36, 51-58).

Thrombin induces a myofibroblast phenotype resistant to apoptosis

The appearance of myofibroblasts in areas of active fibrosis, together with the results of in vitro studies, strongly suggests that myofibroblasts are key contributors to the increased extracellular matrix synthesis characteristic of SSc and other interstitial lung fibroses (8-10, 35, 59). Myofibroblasts can be cultured from BAL fluids of scleroderma patients with interstitial fibrosis (57). Previously, we reported that such cells express more collagen I, collagen III and fibronectin than do normal lung fibroblasts, and that they have an enhanced proliferative response upon ex-
Normal and SSc lung fibroblasts were grown to 80% confluency and then stimulated with Camp (cAMP 16 μM), Cer (ceramide 6, 20 μM) and FasL (20 ng/ml) in the presence or absence of Thr, thrombin (0.5 U/ml) in serum-free medium for 24 hours. Control cells were incubated in serum-free medium only. White bars represent normal lung fibroblasts and black bars represent SSc lung fibroblasts. For the flow cytometry analysis, single cell suspensions were prepared by trypsinization. Cells were then fixed in 70% ethanol and stained with propidium iodine (120 μg/ml). Cell fluorescence was measured with a FACS scan flow cytometer. A negative control gate was set using cells incubated in serum-free medium only. A minimum of 10,000 events were collected per sample. Measurement of fluorescence was performed at > 620 nm. Apoptotic cells are expressed as a percentage of the total cells in the population. Thrombin induces resistance to apoptosis induced by camptothecin, ceramide and FasL.

Fig. 2. Thrombin makes normal and sclerodema (SSc) lung fibroblasts resistant to apoptosis.

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N-terminal extracellular domain, converting the inactive receptor to an active form capable of interacting with multiple G proteins, e.g. Gi, Gq, G12/13, in the same cell (75,76,78). Gq-dependent signaling activates phospholipase C, which leads to phosphoinositide hydrolysis and results in Ca\(^{2+}\) mobilization and activation of protein kinase C (PKC). PKC is required for DNA synthesis as well as smooth muscle cell growth (72), both necessary events in thrombin-mediated cell proliferation (72,75,76). G\(\beta\)\(\gamma\) subunits of Gi-protein activate phosphoinositide-3-kinase (PI-3K) followed by Akt phosphorylation, which protects cells from apoptosis (64,68,69). The \(\alpha\) subunit of G12 and G13 binds Rho guanine-nucleotide exchange factors, activating small G-protein RhoA and mediating cytoskeletal reorganization (79-81). Our recent studies demonstrate that the PKCe/MAPK and PI3K/P70S6 kinase pathways represent key signaling routes in thrombin-induced lung fibroblast (82).

**PKC and Rho pathways in thrombin-activated myofibroblasts**

In previous studies we demonstrated that thrombin induces IL-8 and tenascin-C via activation of PAR-1 and downstream through activation of PKC\(\gamma\) and PKC\(\varepsilon\), respectively (34,35). Thrombin-induced differentiation of normal lung fibroblasts to a myofibroblast phenotype is mediated by PAR-1 (27) and by multiple downstream signaling pathways such as PKC and Rho (27,28) (Fig.3). Depletion of PKC\(\varepsilon\), inhibition of PKC\(\varepsilon\) or inhibition of Rho activation abolishes thrombin-induced SM-\(\alpha\) expression/organization and collagen gel contraction by lung fibroblasts (27,28), suggesting a role for each in myofibroblast differentiation. The molecular link between Rho and actin stress fiber formation has recently been identified (79). The downstream target of Rho, Rho-kinase, directly phosphorylates the myosin light chain, promoting interaction of myosin filaments with actin filaments, followed by stress fiber formation and increased contractility (83). Thrombin has been shown to promote actin reorganization in endothelial and astrocytoma cells via Rho-dependent activation of myosin light chain, but without PKC involvement (79,83). Our results indicate that thrombin rapidly activates Rho and significantly increases Rho-\[^{35}\text{S}\]GTP\(\gamma\)S binding in lung fibroblasts (28). Thrombin strongly stimulates Rho activity \textit{in vivo} and initiates PKC\(\varepsilon\)-RhoA complex formation (28). The Rho inhibitor, toxin B, which inactivates Rho by ADP ribosylation, inhibits thrombin-induced SM-\(\alpha\) expression/organization and collagen gel contraction; toxin B also inhibits PKC\(\varepsilon\)/SM-\(\alpha\) actin complex formation and PKC\(\varepsilon\)/RhoA co-immunoprecipitation by lung fibroblasts (28). Moreover, we have demonstrated that PKC\(\varepsilon\)/RhoA complex formation is an early event in thrombin’s activation of lung fibroblasts (28). Using a yeast two-hybrid system, direct interactions between the yeast homologues of Rho protein, Rho1p, and the homologues of mammalian PKC, Pkc-
1p, have been shown (83). Recent studies also provide evidence that mammalian PKC isozymes and Rho GTPases co-immunoprecipitate and participate in direct protein-protein interactions (84). The association of PKCα with Rho has been demonstrated in endothelial cells, suggesting a critical role in Rho activation (85, 86). However, we have observed that Rho protein is not necessary for thrombin-induced PKCε activation and translocation to the membrane, nor does depletion of PKCε affect Rho activation and thrombin-induced GTPgS binding (28). On the other hand, Rho inhibition prevented co-immunoprecipitation of PKCε with SM-α-actin, suggesting that the association of thrombin-activated Rho with PKC is essential for the regulation of SM-α-actin organization in lung fibroblasts (28).

We recently found that overexpression of both constitutively active PKCε and constitutively active RhoA induces highly organized SM-α-actin and the contraction of collagen gels; individually, neither was capable of these functions (28). Studies have shown that Rho alone can increase the activity of SM-α-actin promoter and modulate SM-α-actin expression in other cell types, such as vascular smooth muscle cells (81). However, our data suggest that PKCε, in association with RhoA, recruits SM-α-actin and possibly some other protein(s) to promote the intracellular events responsible for SM-α-actin expression and organization. Thus, PKCε- and Rho-mediated signaling pathways are essential for thrombin-induced differentiation of normal lung fibroblasts to the scleroderma myofibroblast phenotype. Based on our recent observations and data from the literature, we have proposed a hypothetical mechanism for the development of myofibroblast differentiation leading to pulmonary fibrosis (see Figure 4).

Thrombin and microvascular alterations in pulmonary fibrosis
Microvascular changes, such as the enhanced proliferation of endothelial cells and neovascularization, are observed in lung fibrosis (87-98). Few studies, however, have addressed the importance of vascular remodeling in the lung during the course and development of pulmonary fibrosis. We have observed the proliferation of microvascular endothelial cells and the generation of functional capillaries in scleroderma lung fibrosis (Fig. 1). Early vascular dysfunction in scleroderma lung may result from the hyperproliferation of endothelial cells and possibly from the increased numbers of alveolar capillaries (hypervascularity). These microvascular alterations are seen most prominently during early phases of pulmonary fibrosis (Fig. 1) Similarly, enhanced proliferation of endothelial cells and neovascularization mediated by IL-8 and IL-10 have been observed in IPF (89), as well as in bleomycin-induced pulmonary fibrosis in mice (90).

Thrombin has been shown in vitro and in vivo to promote endothelial cell activation followed by angiogenesis, and this also is mediated by PAR-1 and via PKC (73). Interestingly, abrogation of thrombin-induced increases in pulmonary microvascular permeability has been shown in PAR-1 knockout mice (98). The authors suggest that PAR-1 is critical in mediating the permeability-increasing and vasoconstrictor effects of thrombin in pulmonary microvessels (98). Moreover, thrombin-stimulated lung fibroblasts secrete several proan-

**Fig. 4.** Proposed schema for thrombin-activated lung fibroblast and differentiation to a myofibroblast phenotype resistant to apoptosis. Thrombin, via its proteolytically activated receptor PAR-1, activates PKCα, PKCε and RhoA. Activation of PKCε and RhoA results in PKCε/RhoA immunocomplex formation followed by the formation of a complex with SM-α actin (SMA). Ternary complex PKCε/RhoA/SMAreresults in significantly increased SM-α actin expression and organization, causing differentiation of normal lung fibroblasts to a myofibroblast phenotype. Activated PKCε and RhoA inhibit FasL-induced apoptosis, resulting in transformation of the lung fibroblast to a phenotype that is resistant to apoptosis. Additionally, activated PKCε mediates thrombin-induced DNA synthesis in lung fibroblasts and participates in the differentiation of these cells. Factors induced by thrombin in lung fibroblasts, e.g. PDGF, mediate DNA synthesis, while tenascin C (TN), fibronectin (FN) and connective tissue growth factor (CTGF) inhibit apoptosis in lung fibroblasts and are involved in the induction of resistance to apoptosis.
giogenic factors, e.g. metalloproteinases (MMP’s), which increase endothelial cell invasion, and VEGF, VCAM-1, IL-8, PDGF, PAI-1 and tenasin-C (91, 93-96, 99). As noted above, thrombin induces CTGF in fibroblasts, promoting endothelial cell proliferation and neovascularization in vivo (51, 55). Thus, thrombin induces an angiogenic phenotype in microvascular endothelial cells in vitro and promotes angiogenesis in vivo. The angiogenic action of thrombin via PAR-1 has also been demonstrated in tumor progression and metastases, including in lung (98). The presence of thrombin in many disease conditions in which neovascularization is activated, such as inflammation, atherosclerosis and cancer, as well as in lung fibrosis, suggest a pivotal role for thrombin in the progression of such diverse pathological conditions (37).

Role of thrombin in cross talk among activated lung fibroblasts, epithelial cells and endothelial cells during the development of pulmonary fibrosis

During the development of lung fibrosis, activated fibroblasts interact with other cell types such as immune cells (macrophages, monocytes, T cells, mast cells and eosinophils) and non-immune cells (epithelial and endothelial cells) (8-10, 95, 100, 101). There is now considerable evidence that myofibroblasts are capable of modulating the properties of other cells through the paracrine activity of soluble product(s) (49, 50, 95). Factors secreted by endothelial cells are mitogenic for various mesenchymal cells and stimulate collagen production (53-55). Factors secreted by both cell types promote the migration of these cells to various areas of tissue injury in the lung (100, 101). Because thrombin activates lung fibroblasts and increases the secretion of proangiogenic factors from these cells, as well as from endothelial cells, the interaction of these two cell types in the presence of thrombin may be crucial in the development of lung fibrosis. Similarly, factors secreted by epithelial cells are mitogenic for various mesenchymal cells (fibroblasts and smooth muscle cells) and stimulate collagen production (10, 95). Epithelial-mesenchymal transformation (EMT), besides its role in embryonic development, tumorigenesis and organ remodeling during fibrogenesis (101), has been shown to participate in the progression of lung fibrosis. Inflammatory factors known to participate in epithelial cell and fibroblast differentiation, such as TGF-β1, IL-8, IL-11 and fibronectin, are induced by thrombin in several different cell types (27, 28, 37, 100, 101). Because all of these cell types utilize similar signaling pathways when activated by thrombin, targeting PAR-1 and/or its downstream signaling molecules may prove to be a powerful approach to the treatment of pulmonary fibrosis.

Thrombin-induced signaling as a potential therapeutic target in lung fibrosis

In an animal model of pulmonary fibrosis, a direct thrombin inhibitor, UK-156406, attenuates lung collagen accumulation by lowering the profibrotic effects of thrombin and suppressing CTGF synthesis (36, 49). This peptide inhibits thrombin’s proteolytic activity by binding in its catalytic triad (49). Other direct thrombin inhibitors, e.g. lepirudin, bivalirudin, argatroban and melagatan, also interact with thrombin by blocking its catalytic activity, and are currently approved for clinical use in cardiovascular disease (102). Another even more attractive way of targeting thrombin’s action is by the inhibition of the thrombin receptor. The collection of thrombin receptor antagonists currently available falls into several categories: (i) peptide antagonists; (ii) peptidomimetics; (iii) non-peptide thrombin receptor inhibitors; and (iv) statins (103-106). Several peptides with structural similarities to the tethered ligand have been shown to prevent thrombin-induced cellular effects (103), but in vivo studies are still very limited. Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, suppress tissue factor, and thus thrombin generation. It has been suggested that statins inhibit PAR-1 expression and desensitize cells to thrombin stimulation (104). Several investigators have demonstrated that statins downregulate the prenylation of the proteins involved in signal transduction, including PKC and Rho (104-106). Selective inhibition of the profibrotic effects of thrombin or PAR-1 by direct thrombin inhibitors or PAR-1 antagonists at the cellular level may avoid potential complications, e.g. thrombocytopenia or thrombosis, associated with the inhibition of thrombin and other coagulation proteases. We believe that targeting thrombin and/or PAR-1 signaling may represent an attractive therapeutic approach for SSc-associated lung fibrosis, as well as other forms of pulmonary fibrosis.

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