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STRATEGIES FOR ANTIFIBROTIC THERAPIES

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Abstract

The fibrotic diseases encompass a wide spectrum of entities including such multisystemic diseases as systemic sclerosis, nephrogenic systemic fibrosis and sclerodermatous graft versus host disease, as well as organ-specific disorders such as pulmonary, liver, and kidney fibrosis. Collectively, given the wide variety of affected organs, the chronic nature of the fibrotic processes, and the large number of individuals suffering their devastating effects, these diseases pose one of the most serious health problems in current medicine and a serious economic burden to society. Despite these considerations there is currently no accepted effective treatment. However, remarkable progress has been achieved in the elucidation of their pathogenesis including the identification of the critical role of myofibroblasts and the determination of molecular mechanisms that result in the transcriptional activation of the genes responsible for the fibrotic process. Here we review the origin of the myofibroblast and discuss the crucial regulatory pathways involving multiple growth factors and cytokines that participate in the pathogenesis of the fibrotic process. Potentially effective therapeutic strategies based upon this new information are considered in detail and the major challenges that remain and their possible solutions are presented. It is expected that translational efforts devoted to convert this new knowledge into novel and effective antifibrotic drugs will be forthcoming in the near future.

Keywords: Fibrotic Diseases, Myofibroblast, Fibrosis, Tyrosine Kinases, TGF- β , Antifibrotic Therapy

The Problem

Fibrotic diseases encompass a wide spectrum of entities including such multisystemic diseases as systemic sclerosis (SSc), sclerodermatous graft versus host disease, and nephrogenic systemic fibrosis, as well as organ-specific disorders such as pulmonary, liver, and kidney fibrosis (1-8). Although their etiology and causative mechanisms vary widely, these conditions share the common feature of elevated expression of genes encoding matrix proteins, and the resulting fibrosis disrupts the normal architecture of the affected organs, ultimately leading to their dysfunction and failure (9-11). Indeed, it is the persistent production of extracellular matrix (ECM) macromolecules by activated mesenchymal cells, known as myofibroblasts, that distinguishes controlled repair occurring during normal wound healing from the uncontrolled fibrosis that is the hallmark of fibrotic diseases.

Collectively, given the wide variety of affected organs, the chronic nature of the fibrotic processes, and the large number of individuals suffering their devastating effects, these diseases pose one of the most serious health problems in current medicine and represent an enormous challenge to health services, and a serious economic burden to society. It has been estimated that as much as 45% of the mortality in the Western developed countries is now caused by fibrotic diseases (11) and the mortality in underdeveloped or developing countries caused by these diseases is likely to be even much higher. Despite these considerations there is currently no accepted effective treatment. However, recent identification of the critical role of the myofibroblast and elucidation of crucial regulatory pathways resulting in the transcriptional activation of the genes encoding collagens and other proteins responsible for the fibrotic process has been of substantial importance and has provided a sound basis for the development of novel and effective means of therapy. Unfortunately, the current lack of approved drugs for treatment

of these diseases indicates that recent knowledge has not yet been translated into effective therapies, however, it can be expected that this situation will be reversed in the near future.

Origin and Critical Role of Activated Fibroblasts/Myofibroblasts in Fibrotic Tissues

Although the causative agent or initiating event of the fibrotic disorders are quite diverse, and their pathogenesis is variable, a common feature in affected tissues is the presence of large numbers of activated fibroblasts or myofibroblasts. These cells display unique biological functions, including increased production of fibrillar type I and type III collagens, expression of α -smooth muscle actin (α -SMA), a molecular marker of activated myofibroblasts, and reduction in the expression of genes encoding ECM-degradative enzymes (12-15). Regardless of the etiological event, the accumulation of myofibroblasts in affected tissues and the uncontrolled persistence of their elevated biosynthetic functions are crucial determinants of the extent and rate of progression of the fibrotic diseases, and of their clinical course, response to therapy, prognosis, and mortality (9).

The origins of myofibroblasts are diverse and may differ depending on the affected organ and the initiating event. There are four important sources of the myofibroblasts encountered in the fibrotic tissues as illustrated in Figure 1: i) Proliferation and activation of tissue resident fibroblasts or perivascular and vascular adventitial fibroblasts in response to specific signals from infiltrating inflammatory cells resulting in activation of quiescent fibroblasts to a myofibroblast phenotype (16). ii) Recruitment of fibroblast precursor cells from bone marrow through local release of activated chemokines. These circulating bone marrow precursor cells are known as fibrocytes, a unique cell population expressing bone marrow cellular surface markers, such as CD34 protein, but capable of ECM production. These cells can migrate from the

bloodstream in response to specific chemokine gradients and localize in tissues undergoing pathological fibrogenesis (17-18). iii) Transdifferentiation of epithelial cells to myofibroblasts, a process known as epithelial to mesenchymal transition (EMT), which is induced by transforming growth factor β (TGF- β) and perhaps other polypeptides such as endothelin-1 (ET-1) or insulin growth factor (19-21). In EMT, epithelial cells lose their epithelial characteristics, including E-cadherin expression and apical-basal polarity, and reorganize their cytoskeleton to acquire a motile behavior and the phenotype of myofibroblasts including the expression of α -SMA and fibroblast-specific proteins such as type I collagen. Although some recent studies employing genetic cell lineage tracing studies have raised controversy (22), numerous publications have described the occurrence of EMT in the course of renal, pulmonary, and liver fibrosis (21,23-25). iv) Another type of cellular transition similar to EMT, but in which endothelial cells undergo a mesenchymal transition (EndoMT) has emerged as a possible mechanism in pathological fibrosis (26,27). During EndoMT endothelial cells lose their specific endothelial cell markers, such as vascular endothelial cadherin (VE cadherin), and acquire a mesenchymal or myofibroblastic phenotype initiating expression of α -SMA, vimentin, and type I collagen. In addition, these cells also become motile and are capable of migrating into surrounding tissues. Similar to EMT, EndoMT can be induced by TGF- β (26,27).

Whereas investigations into the pathogenesis of fibrotic diseases have been ongoing for many decades, discoveries within the last ten years have more fully characterized the molecular complexity of the processes involved. Since its first identification in 1983 by Sporn and collaborators (28-30) it has been known that transforming growth factor- β (TGF- β) a pleiotropic growth factor involved in the pathogenesis of numerous human diseases, plays a key role in fibrotic diseases by stimulating the production of various collagens and other ECM components

by activated mesenchymal cells (29,31-33). TGF- β also inhibits the synthesis of matrix degrading metalloproteinases and stimulates the production of tissue inhibitors of metalloproteinases (34). However, multiple recent findings have clearly identified numerous other cytokines including interleukins 4, 6, and 13 and several novel signaling pathways such as the Wnt, Hedgehog and Notch as potential participants. Several other important new insights have been made which have a critical bearing on therapeutic approaches for the fibrotic diseases. There is a growing realization that the clinical manifestations and the molecular pathogenesis of a given fibrotic disease are highly heterogeneous. As discussed above, besides the variety of causes, often poorly understood, which may be responsible for initiating the fibrotic response there is also increasing complexity at the cellular and molecular levels. Despite this heterogeneity, however, the final common result is the excessive production of ECM. Thus, the focus of this review will be on the cellular alterations and molecular pathways that play critical roles in the fibrotic response and to discuss potential strategies that may allow identification of potential therapeutic targets.

Critical Molecular Pathways Participating in the Fibrotic Response

Transforming Growth Factor Beta (TGF- β)

The TGF- β s comprise three structurally similar proteins, TGF- β 1, TGF- β 2 and TGF- β 3, with a wide and diverse spectrum of biological activities ranging from a role in embryonic development to regulation of the immune response and to the development of exaggerated fibrotic responses (35,36). Whereas deregulated TGF- β signaling occurs in essentially all fibrotic reactions, the specific mechanisms vary depending on the causative agent(s) and on the affected organ. TGF- β , initially produced as an inactive homodimeric peptide by numerous cell types,

including fibroblasts and macrophages, is secreted into the ECM as a large latent complex (Figure 2). The latent TGF- β complex consists of latent TGF- β binding protein covalently bound to a small latency complex formed by a homodimer of TGF- β which is bound to an RGD-containing N-terminal latency associated binding peptide (37,38). TGF- β can undergo several alternative proteolytic or conformational activating events (39,40). Once activated TGF- β binds to a constitutively active serine/threonine transmembrane receptor kinase known as TGF- β receptor II (T β RII). The intracellular transduction pathways following TGF- β binding to its cognate receptors are quite complex and involve either the Smad pathways or non-Smad signaling pathways. The Smad pathways are often referred to as the canonical pathway whereas the non-Smad pathways are referred to as the non-canonical pathways.

Canonical TGF- β signaling pathways.

In the classic or canonical pathway the ligand-bound T β RII recruits and phosphorylates a TGF- β receptor I (T β RI). The T β RI comprise a family of proteins also known as ALK proteins, two of which, ALK-1 and ALK-5, are involved in TGF- β signaling (41-43). ALK-5 is the most common T β RI and is phosphorylated by T β RII on three to five serine and threonine residues in a short 30 amino acid regulatory sequence. Signaling from the T β RI to the nucleus then occurs through the receptor activated RSmads, Smad2 or Smad3 which are phosphorylated by T β RI. The phosphorylated Smad2/Smad3 then bind to the co-Smad, Smad4, forming a complex that translocates across the nuclear membrane (44-48). In the nucleus the Smad complexes in association with co-activators and co-repressors and other transcription factors modulate the expression of target genes as illustrated in Figure 2. Fine tuning of TGF- β activity is achieved through a balance of positive and negative effector molecules. Of critical importance is the inhibitory Smad, Smad7, which inhibits TGF- β signaling through binding to T β RI receptor,

preventing recruitment and phosphorylation of RSmads and also facilitating T β RI degradation, leading to inhibition of RSmad activation (49).

Non-Canonical TGF- β signaling pathways. In addition to the Smad pathway, critical profibrotic signaling cascades independent of RSmads can be activated in a cell-specific and context-dependent manner and mediate important TGF- β effects (50,51). Of great importance with respect to the fibrotic effects of TGF- β is the finding that TGF- β stimulation leads to the activation of PI3K, which in turn activates two important profibrotic pathways: p21 activated kinase (PAK2)-Abelson kinase (Abl) and Akt-mTOR1 pathways. Downstream targets of c-Abl include known profibrotic mediators such as PKC δ /Fli-1, Egr, and Smad1 (52-55) as illustrated in Figure 2. Activated c-Abl is required for the activation of Smad1 and for the phosphorylation of protein kinase C- δ (PKC- δ), which in turn phosphorylates the transcription factor Fli-1 reversing its inhibitory effect on collagen gene expression. Furthermore, recent studies have shown that the c-Abl-PKC δ pathway participates in the process of endothelial-mesenchymal transition (26). The activation of Smad1 downstream of TGF- β is of particular interest since total and phosphorylated Smad1 levels were significantly elevated in SSc skin biopsy samples and in cultured SSc fibroblasts (54). The Akt-mTOR pathway plays an important role in various cell processes including regulation of cell proliferation and metabolism as well as being involved in some epithelial/mesenchymal transitions (56). Another important non-Smad signaling pathway is through the activation of Jun N-terminal kinase (JNK) resulting in the activation of c-Jun, a critical profibrotic transcription factor (50,51). In addition to serine/threonine phosphorylation, T β RI can also be phosphorylated on tyrosine residues in response to TGF- β activation leading to activation of Erk1/2 MAPK, which can play an important role by regulating myofibroblast

formation as well as matrix synthesis (57-60). Other studies have shown that tyrosine phosphorylation of T β RII, triggers activation of p38 MAPK signaling (61,62).

Connective tissue growth factor (CTGF/CCN2).

CTGF, also known as CCN2, is another pleotropic growth factor that has recently emerged as an important mediator of normal and pathological tissue fibrotic responses (63,64) and it has been suggested to play a crucial role in SSc tissue fibrosis (65,66). *In vitro* studies in cultured fibroblasts have shown that the addition of recombinant CTGF or its overexpression induced fibrosis. Furthermore, in experimental mouse models, elevated levels of CTGF were associated with the development of fibrosis, and suppression of CTGF reduced bleomycin-induced lung fibrosis (67). TGF- β stimulates CTGF synthesis in fibroblasts, vascular smooth muscle cells and endothelial cells, and several studies have shown that CTGF acts as a downstream mediator to enhance the fibrogenic action of TGF- β (68). Indeed, the CTGF produced by these cells in response to TGF- β stimulation in turn stimulates the synthesis of such ECM components as type I collagen and fibronectin in fibroblasts and very likely also in endothelial cells (63,64). Significantly, Smad1 activation correlated with elevated CTGF protein and CTGF promoter activity and DNA binding assays demonstrated that Smad1 was a direct activator of the CTGF gene (54). Of considerable interest is the finding that imatinib mesylate, an inhibitor of c-Abl, blocked activation of the Smad1 pathway in TGF- β stimulated normal fibroblasts and abrogated stimulation of CTGF expression in SSc fibroblasts (54). These observations indicate that, at least in some circumstances, c-Abl is required for Smad-1 activation. It should also be noted that CTGF expression can be independently stimulated through the RhoA/ROCK pathway (69) as illustrated in Figure 2. Currently, trials testing anti-CTGF antibodies in interstitial pulmonary fibrosis (IPF) and liver fibrosis are ongoing.

Platelet derived growth factor

The platelet derived growth factor (PDGF) family consists of four different polypeptides, the original PDGF-A and PDGF-B, and the more recently described PDGF-C and PDGF-D. The biologically active PDGFs form disulphide-bonded dimers, such as PDGF-AA and PDGF-BB as well as a PDGF-AB heterodimers. The PDGFs bind and activate two structurally related tyrosine kinase receptors, PDGFR α and PDGFR β . Ligand-induced receptor homo- or heterodimerization leads to autophosphorylation of specific tyrosine residues within the cytoplasmic domain. PDGF-A activates PDGFR α , exclusively, whereas PDGF-B is capable of activating PDGFR α , PDGFR $\alpha\beta$ and PDGFR β . PDGF-AB can activate PDGFR α and PDGFR $\alpha\beta$ (70-72). Receptor activation leads to overlapping signal transduction pathways including PI3K, Ras-MAPK, Src family kinases and phospholipase C γ (PLC γ) resulting in various cellular responses including proliferation, chemotaxis and actin reorganization as illustrated in Figure 3. Multiple reports have implicated PDGF in fibrotic reactions of several organs, including pulmonary, renal and hepatic fibrosis as well as in SSc (73). Fibroblasts are both major sources and targets for PDGF-A since they express PDGFR α on their cell surface (74-76). Thus, PDGF-A/PDGFR α signaling loops can stimulate fibroblasts to synthesize ECM and release pro-fibrotic mediators. PDGF-B is primarily released by macrophages and hepatic stellate cells, with the latter ones also pointing to a major role of PDGF-B/PDGFR β -signaling in liver fibrosis (77,78). Upon tissue injury PDGF signaling becomes activated to promote wound closure but PDGF signaling is tightly regulated and turned off as soon as the physiologic repair processes are completed (79). Failure to terminate activated PDGF signaling may lead to excessive scar formation and tissue fibrosis.

Wnt-Signaling

While β -catenin has a structural role linking transmembrane cadherins to the actin cytoskeleton, it also plays a critical role in canonical Wnt signaling. In the absence of Wnt signals, β -catenin binds to and is phosphorylated by a complex consisting of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3 β (GSK-3 β), and casein kinase which promotes subsequent degradation of β -catenin. Wnt proteins are secreted ligands that transmit their signal across the plasma membrane by interacting with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP5/6) as illustrated in Figure 3 (80). Wnt receptor binding disrupts the complex resulting in the stabilization of β -catenin which translocates to the nucleus, where it binds to T-cell factor/lymphoid enhancer-binding factor (Tcf/Lef) to induce target gene transcription such as axin-2 (81). Aberrant activation of the canonical Wnt signaling pathway has been implicated in a variety of pathological processes including pulmonary, renal, dermal and liver fibrosis, as well as, in scarring following myocardial infarction and fibrosis accompanying muscular dystrophy (82-86). Wnt signalling is tightly controlled by an array of negative regulators, among which, Dickkopf proteins (Dkk-1-4) have a key role. The best studied is Dkk-1, which functions as a natural secreted antagonist of Wnt signaling (87,88).

Hedgehog Signaling

Mammalian orthologs of the *Drosophila melanogaster* hedgehog (Hh) morphogen are highly hydrophobic secreted peptides and three different hedgehog proteins have been described, Sonic hedgehog (Shh), Indian hedgehog, and Desert hedgehog with Shh being the most important in the present context (89). Patched (Ptc), a twelve-pass membrane protein binds Hh ligands, but in the absence of ligand, Ptc interacts with and inhibits Smoothed (Smo), a seven-pass membrane protein (90). However, binding of Shh to Ptc1 induces conformational changes

that prevent Ptc1 from inhibiting Smo. The absence of Smo inhibition initiates a series of intracellular events resulting in stabilization of Gli family zinc finger transcription factors which in turn stimulate expression of Hh target genes (91) as illustrated in Figure 3. While Hh signaling is critical during embryonic development, inappropriate activation has been implicated in the pathogenesis of various diseases in adults, including a variety of malignancies (92-94). A recent study has shown that overexpression of Shh in cultured SSc fibroblasts activates the Hh pathway, with accumulation of the transcription factor Gli-2 and increased expression of Hh target genes (95). Furthermore, Shh potently stimulated the production of collagen and induced the differentiation of resting fibroblasts into myofibroblasts. Overexpression of Shh in the skin of mice was sufficient to induce fibrosis, and mice lacking one allele of the gene for the inhibitory receptor Ptc1 were more sensitive to experimentally-induced fibrosis (95).

Notch Signaling

Notch signaling, also first discovered in *Drosophila*, is initiated by binding of members for two ligand families, Jagged and Delta-like. Binding of ligands such as Jagged-1 (Jag-1) results in cleavage of Notch receptors by the γ -secretase complex and release of the active Notch intracellular domain (NICD) as illustrated in Figure 3 (96,97). Translocation of the NICD into the nucleus activates the transcription of numerous target genes such as the Hairy/Enhancer of Split (Hes) (98). Abnormal Notch signaling participates in the pathogenesis of several human diseases including T-cell acute lymphoblastic leukemia and melanoma (99,100). Clinical trials with γ -secretase inhibitors already in progress in patients with T-cell acute lymphoblastic leukemia have yielded promising results (101). There is accumulating evidence for the

importance of Notch signaling in fibrotic diseases, although the molecular mechanisms involved in fibroblast activation and stimulation of ECM synthesis need clarification (102,103).

Endothelin-1

Endothelin-1 (ET-1) is a 21-amino acid polypeptide with potent vasoconstrictor activity that plays a crucial role in the pathophysiology of pulmonary arterial hypertension and has been identified as a prime therapeutic target for this disorder (104,105). A growing body of evidence has implicated ET-1 as a participant in organ fibrosis and numerous studies have described a variety of its profibrogenic activities. ET-1 has been shown to stimulate the synthesis of extracellular matrix macromolecules such as collagen types I and III and to inhibit the production of matrix degrading metalloproteinase-1 in cultured normal human fibroblasts (106,107). Furthermore, increased production of ET-1 has been demonstrated in a variety of human fibrotic diseases (108) and in experimentally induced pulmonary fibrosis (109) providing strong support to the concept that ET-1 also plays an important role in the pathophysiology of SSc, pulmonary fibrosis and other fibrotic diseases (110-112). ET-1 also was found to be involved in the induction of EMT (113). The role of ET-1 in EMT appears to be mediated through the endothelin A receptor (ET-A) via stimulation of endogenous TGF- β 1 production (114).

Strategies for Inhibition of Fibrosis

Cellular phenotypic transitions resulting in myofibroblast formation. Recent delineation of the cellular phenotype transitions leading to the activated myofibroblast and the molecular identification of the pathways stimulating the fibrogenic processes have suggested new therapeutic approaches and targets. As discussed above and shown in Figure 1, there are four distinct potential cell sources of myofibroblasts. Three of these may be found as residents in the

affected tissue including fibroblasts, endothelial cells and epithelial cells. Thus blocking their transition into activated myofibroblasts could prevent the initiation of the fibrotic process itself. The most direct transition is the conversion of fibroblasts into myofibroblasts in which the activated cells express α -SMA and synthesize markedly increased amounts of ECM components. Undoubtedly, TGF- β is the most important causative agent involved in the fibroblast activation process as well as the mesenchymal transformation of epithelial and endothelial cells. Thus, preventing TGF- β action is clearly a potential therapeutic approach that may be achieved at several levels.

Interfering with TGF- β expression and activation. The complex signaling pathways initiated by TGF- β receptor binding offer multiple points of potential therapeutic intervention. TGF- β is constitutively synthesized and stored in an inactive form as a complex with specific binding proteins. Thus, the expression of TGF- β and the process of its release in an active form present therapeutic targets. Pirfenidone has been studied in experimental models of pulmonary fibrosis in which it suppressed TGF- β gene expression at the transcriptional level and significantly reduced the tissue levels of TGF- β mRNA and TGF- β in lavage fluid (115). Pirfenidone has been evaluated in four randomized, double-blind, placebo-controlled clinical trials. The collective results of these trials indicate that pirfenidone can reduce the rate of decline in lung function as measured by changes in forced vital capacity or total lung capacity but had little effect on diffusing capacity or resting arterial oxygen levels (116,117)

Several α v integrins can activate matrix-bound latent TGF- β and antibodies to the integrin α v β 6 expressed on epithelial cells blocked the activation of TGF- β preventing development of experimental lung fibrosis (118-120). Such a specific antibody approach has the

advantage of confining the blocking activity only to injury sites where $\alpha v\beta 6$ is expressed only in epithelia and not compromise other important functions of TGF- β .

Angiotensin II in certain circumstances exhibits remarkable profibrotic properties probably acting, at least in part, through stimulation of TGF- β production (121,122). Although angiotensin converting enzyme and AT1R inhibitors can block experimental models of lung fibrosis, there are no published reports regarding the use of these drugs in humans with pulmonary fibrosis. Therefore, it is still unclear whether ATII inhibition would have beneficial effects in fibrotic conditions, although the complex interactions between TGF- β and ATII suggest that pharmacologic inhibition of both systems may yield synergistic beneficial effects.

Blocking or inactivating extracellular TGF- β is a possible approach but results to date in humans have so far not been promising even though a TGF β 1 inhibitor peptide attenuated the progression of lung fibrosis in the bleomycin pulmonary fibrosis model (123). In a phase I/II double-blind, placebo controlled trial of CAT-192, a human anti-TGF β 1 monoclonal antibody in patients with early diffuse SSc no improvement was found in the extent or severity of skin involvement or in lung function parameters (124). Also because of the wide-ranging functions of TGF- β , interfering globally with this essential signaling molecule could have untoward, toxic effects as was the case in the CAT-192 clinical trial (124).

Inhibition of homing of circulating profibrotic cells. Circulating fibrocytes which express the CXCR4 receptor migrate to fibrotic sites in response to CXCL12 chemokine gradients and the CXCL12/CXCR4 axis appears to be up-regulated in SSc (125). CXCL12 antagonizing antibodies as well as AMD3100, a CXCR4 antagonist significantly reduced bleomycin-induced lung fibrosis (126,127). AMD3100 is approved for the treatment of some

malignancies such as non-Hodgkin's lymphoma, thus targeting the CXCL12/CXCR4 axis with available drugs represents a potentially effective anti-fibrotic therapeutic strategy.

Various experimental studies as well as *ex vivo* human data support a pro-inflammatory and pro-fibrotic role for the monocyte chemoattractant protein-1 (MCP-1/CCl-2). Inhibition of MCP-1 signaling by blocking its receptor, CCR2, reduced the accumulation of ECM proteins in animal models of skin and lung fibrosis (128). Since antagonists of CCR2 are currently in clinical trials for various diseases, they may also be evaluated as anti-fibrotic agents.

Blocking TGF- β signaling pathways (see Figure 2).

Inhibition of TGF- β receptor and canonical signaling. There have been several studies of specific inhibitors of TGF- β type I receptor and the most thoroughly studied is SM305 which proved to have excellent selectivity and potency against ALK5 and ALK4. In normal dermal fibroblasts, SM305 abrogated TGF- β -induced ECM gene expression, fibrogenic cytokine production, Smad3- and Smad2-dependent transcriptional responses, and fibroblast transdifferentiation into myofibroblasts (129). These inhibitory effects of SM305 were associated with potent selective suppression of TGF- β -induced phosphorylation and nuclear translocation of R-Smads. However, in unstimulated SSc fibroblasts, SM305 only caused variable and modest reduction in levels of type I collagen, and did not reverse constitutive Smad nuclear accumulation or the proportion of α -SMA-positive myofibroblasts. Together, these results indicate that ALK5-Smad signal transduction plays a fundamental role in mediating TGF- β -dependent profibrotic responses in normal fibroblasts, whereas Smad nuclear accumulation in SSc fibroblasts appears to be ALK5 independent. In light of its potent antifibrotic activities in normal dermal fibroblasts *in vitro* and *in vivo*, SM305-mediated ALK5 inhibition represents a

novel therapeutic strategy for controlling some TGF- β driven fibrotic conditions. The apparently contradictory results in SSc fibroblasts emphasize the complexity and heterogeneity of fibrotic reactions and the requirement to appropriately select therapeutic approaches to specific diseases.

Specific inhibition of Smad3. SIS3 is a potent and selective inhibitor of Smad3 that has been shown to block the phosphorylation of Smad3 induced by TGF- β without affecting the phosphorylation of Smad2 (130). SIS3 also inhibited the interaction of Smad3 with Smad4 as well as the stimulation of collagen production by TGF- β by normal fibroblasts and their transition to myofibroblasts. Significantly, SIS3 completely blocked the constitutive phosphorylation of Smad3 as well as the increased type I collagen expression in SSc fibroblasts. The effects of SIS3 on EndoMT and on experimentally-induced diabetic nephropathy were studied recently (131). EndoMT was induced in a mouse microvascular endothelial cell line (MMEC) through the use of advanced glycation end products (AGEs) and diabetic nephropathy was induced in mice by administration of streptozotocin (STZ) (124). SIS3 abrogated EndoMT in the MMEC, and reduced renal fibrosis and retarded progression of nephropathy in the STZ-treated mice. Together, these studies suggest that SIS3 may prove to be an effective anti-fibrotic agent in selected diseases.

Selective inhibition of TGF- β non-canonical signaling pathways. A variety of targets present themselves when viewing the multiplicity of components encompassing the non-canonical pathways initiated by TGF- β . Most prominent among these is the non-receptor tyrosine kinase, cAbl. The role of c-Abl in the fibrogenic process was serendipitously discovered when patients receiving treatment with imatinib mesylate (Gleevec) for chronic myelogenous leukemia (CML) caused by Bcr-Abl translocation had a remarkable reduction of bone marrow

fibrosis (132). Imatinib mesylate is a small molecule that specifically inhibits several tyrosine kinases including c-Abl, PDGFR, c-kit and c-fms by blocking the binding of ATP to the active kinase site. Imatinib mesylate has been shown to be effective in preventing the development of organ fibrosis in the kidney, lung, liver, and skin in several animal models (133-138). As seen in Figure 2 and 3, inactivating c-Abl blocks several downstream effector molecules required for the full TGF- β response, while simultaneous inhibition of PDGF potentiates its anti-fibrotic effect.

Imatinib has been used extensively and successfully in the treatment of CML and gastrointestinal stromal tumors with relatively little toxicity. However, the results in clinical trials of fibrotic diseases have been less clearcut. To date in five small clinical trials and several case reports a total of 108 patients with severe SSc have been treated with imatinib. Encouraging results were reported in 3 of 4 of these studies whereas the fifth study was prematurely terminated for safety reasons (139). In contrast, a recent trial of imatinib in patients with IPF failed to accomplish the primary outcome measures with little beneficial effect on survival or lung function improvement, possibly owing to the short term treatment period of only six months (140). However, in phase II and III clinical trials imatinib had a potent and prolonged beneficial effect on pulmonary arterial hypertension and reversed vascular remodeling even when already established (141).

Second generation and wide spectrum tyrosine kinase inhibitors. A significant fraction of the patients treated in the imatinib anti-fibrotic trials withdrew because of mild to moderate side effects. Dasatinib and nilotinib are second-generation tyrosine kinase inhibitors with improved toxicity profiles that have proven effective in animal models of SSc and both of them require only nanomolar concentrations to obtain strong target inhibition compared to the micromolar concentrations required with imatinib (142). In addition to c-Abl and PDGFR,

dasatinib inhibits sarcoma-tyrosine (src) kinases which regulate c-Abl and are activated by TGF- β and PDGF. A specific inhibitor of src kinases, SU6656, reduced skin fibrosis in experimental models of SSc (143). However, no clinical data is currently available for either of these drugs.

Nintedanib (BIBF 1120) is a potent intracellular inhibitor of tyrosine kinases that is in clinical development for the treatment of IPF and various cancers (144). Its targets include (PDGFR), vascular endothelial growth factor receptors (VEGFR), and fibroblast growth factor receptors (FGFR). Because signaling pathways activated by these tyrosine kinase receptors have been shown to be involved in lung fibrosis (145) and other fibrotic processes their inhibition may slow progression of IPF or liver fibrosis (146-149). In a rat model, such inhibition was shown to prevent the development of bleomycin-induced lung fibrosis when nintedanib was administered before or during the fibrotic phase of the disease (147). In a recently published clinical trial for IPF (150), nintedanib at the highest dose employed produced a significant improvement in the decline of forced vital capacity compared with the placebo group. This dose also resulted in a lower incidence of acute exacerbations as compared with placebo with relatively mild and tolerable side effects. Given the intractable nature of IPF, these results suggest that wide spectrum tyrosine kinases may prove useful in treatment of fibrotic diseases.

Rho-associated kinases

The intracellular mechanisms by which cytokines and growth factors such as TGF- β activate fibroblasts and stimulate ECM production are not completely understood. The small GTPase RhoA plays a central role in cell motility and adhesion through reorganization of the actin cytoskeleton. Activation of receptors switches RhoA from an inactive, GDP-bound conformation to an active GTP-bound conformation, which binds to and activates target

molecules (151). Cellular responses to RhoA are mediated by downstream Rho-associated kinases (ROCK) (152,153), which reorganize the actin cytoskeleton through phosphorylation of several substrates that contribute to the assembly of actin filaments and contractility, molecular events critical for the differentiation of resting fibroblasts into active myofibroblasts. When cultured fibroblasts were treated with TGF- β , inhibition of ROCK with the specific inhibitor Y27632 blocked the formation of myofibroblasts and reduced ECM production without cell toxicity (154). The MAP kinase ERK, identified as a down-stream target of ROCK in SSc fibroblasts, played a critical role in the profibrotic response to TGF- β treatment. It is not certain how ERK mediates its fibrotic effects, but it may regulate Smad3 signaling in certain cells or it may activate the transcription factor Sp1 which can stimulate transcription of the type I collagen genes and which is elevated in fibrotic conditions including SSc (155-158). Interestingly, two DNA intercalators, WP631 and mitoxantrone, have been shown to prevent binding of Sp1 to the COL1A1 promoter in human dermal fibroblasts. Both drugs inhibited basal COL1A1 production and mRNA levels without cytotoxicity or apoptosis and prevented TGF- β stimulation of expression (159). These data suggest that Sp1-DNA intercalators may be an effective approach for the treatment of fibrotic diseases.

Inhibitors of hydroxymethylglutaryl-coenzyme A reductase such as statins, which are widely used to lower serum cholesterol, have been shown to have anti-fibrotic effects (160-162), which might be mediated by inhibition of ROCK. Statins interfere with the geranylgeranylation of membrane RhoA (163,164), which prevents the activation of RhoA, which in turn blocks the activation of Rock. Furthermore, a specific inhibitor of geranylgeranyl transferase 1 inhibited type I collagen gene expression in normal and SSc fibroblasts (165). Thus, the antifibrotic activity of statins supports the antifibrotic effects of ROCK inhibitors. The ROCK inhibitor,

fasudil, has been used in Japan and China for the treatment of coronary and cerebral artery vasospasm (166,167). Considering the very low rate of side effects and the favorable clinical experience in those studies, ROCK inhibitors should be considered as promising candidates for targeted anti-fibrotic therapy.

Caveolin-1

Caveolin-1, the most important structural component of caveolae, is also found in several other cellular sites and is expressed in a variety of cell types including epithelial cells, endothelial cells and fibroblasts. Of interest in the present context is its potential role in regulation of the fibrotic reaction and prominent in this regard is its ability to bind and inhibit a substantial number of kinases (168). High levels of caveolin-1 are found in normal lung fibroblasts, whereas much lower levels are found in lung fibroblasts from fibrotic lungs of SSc and IPF patients (169-171). This reduction in caveolin-1 is associated with increased activity of several signaling kinases (MEK, ERK, JNK, Akt) and increased expression of matrix molecules. An interesting finding is the localization in caveolin-1 of the binding site which functions as a kinase inhibitor (amino acids 82-101) known as caveolin-1 scaffolding domain (CSD). When fused to the C terminus of the Antennapedia internalization sequence, CSD can enter cells with low caveolin- concentrations where, like the intact molecule, it can bind kinases, inhibit their activity and also inhibit collagen expression (171). In caveolin-1 null mice, alveolar walls are thickened, the diameter of alveolar spaces is reduced, and ECM is significantly increased (172-174). In the bleomycin lung fibrosis model caveolin-1 levels are depressed, and systemic administration of CSD blocks the fibrotic process (170), suggesting a causal relationship between low caveolin-1 levels and lung fibrosis.

TGF- β and caveolin-1 signaling interact in several ways. TGF- β inhibits caveolin-1 expression in several types of cells including lung and dermal fibroblasts, while caveolin-1 modifies TGF- β signaling by inhibiting Smad3 phosphorylation and its translocation to the nucleus (175). Caveolin-1 also regulates TGF- β signaling through its effects on the endocytosis of TGF- β ligand-receptor complexes, which are present in both caveolin-1 rich lipid rafts and clathrin-rich early endosomes (176,177). Early endosomal internalization increases TGF- β signaling while caveolin-1-rich lipid raft internalization leads to receptor degradation and inhibition of TGF- β signaling (176). Thus, the loss of caveolin-1 in fibrotic tissues will promote TGF- β signaling by early endosomal internalization.

The above discussion must be viewed with some caution, however, since other reports have found higher levels of caveolin-1 in some SSc dermal fibroblasts than in normal dermal fibroblasts and found that caveolin-1 overproduction enhanced collagen expression while caveolin-1 depletion inhibited collagen expression (178,179). Furthermore, caveolin-1 was found to be a positive regulator of phospho-Smad1 and CTGF, a powerful profibrotic cytokine (178).

In spite of these caveats, the use of CSD as a molecular therapeutic must be considered for treating certain fibrotic conditions, such as in the lung, because caveolin-1 regulates several signaling pathways which may be a more effective approach than use of agents targeting a single specific pathway.

Transcription and other intracellular factor modulation

The c-Abl/PKC δ /Fli-1 pathway. As discussed above, c-Abl has a critical role in mediating the fibrotic response initiated by TGF- β . An important part of this activity is the phosphorylation/activation and nuclear localization of PKC δ . PKC δ then phosphorylates the

transcriptional repressor Fli1 at threonine 312 which promotes its interaction with p300/CREB-binding protein-associated factor and subsequent acetylation. These molecular events cause Fli-1 dissociation from the human $\alpha 2(I)$ collagen gene promoter, resulting in enhanced transcription of the gene (see Figure 2) (180). A breakthrough study on the role of PKC δ in the regulation of collagen gene expression found that selective inhibition of PKC δ markedly decreased the production of collagen by SSc fibroblasts (181,182) and a very specific peptide inhibitor of PKC δ is now being investigated as a protective agent for reperfusion injury after myocardial infarction (183). Previous extensive animal experiments have shown this peptide to be a highly effective and specific inhibitor of PKC δ with minimal toxic effects, suggesting its potential utility in fibrotic disorders.

The AP-1 transcription factor family. The transcription factor AP-1 is a heterodimeric molecule composed of members of the Jun (c-jun, junB and junD) and the Fos family (cFos, FosB, Fra-1 and Fra-2). Currently, the most important of these with respect to fibrotic conditions are c-Jun, JunD, cFos, and Fra-2. TGF- β stimulated the expression of Fra-2 in dermal fibroblasts through ERK signaling and JunD through Smad3 pathway (184). JunD, cJun and cFos were found to be overexpressed in the skin and dermal fibroblasts of SSc patients (185). Interestingly, the effects of AP-1 on collagen synthesis seem not to be restricted to SSc dermal fibroblasts but might also occur in cardiac, renal, and keloid fibroblasts (186-188). These are important findings since they suggest that a critical mode of TGF- β profibrotic activity is through up-regulation of AP-1 and its resultant transcriptional activity. In this regard it should be noted that AP-1 up-regulates tissue inhibitor of metalloproteinases (TIMP) while decreasing matrix metalloproteinase-1 (MMP-1) (189). Thus, AP-1 not only increases the production of matrix components, but also inhibits their degradation resulting in their net accumulation.

T-5224 is a small molecule inhibitor of AP-1 that was developed based on the x-ray crystal structure of the basic region leucine zipper domain of the AP-1-specific DNA complex composed of c-Jun/c-Fos. In a preclinical animal study, T-5224 had an excellent safety profile (190). T-5224 blocked the *in vitro* effects of TGF- β on the differentiation of resting fibroblasts into myofibroblasts and stimulation of ECM production and *in vivo* prevented dermal fibrosis induced by bleomycin (185). Because of the potent antifibrotic effects and the favorable safety profile of T-5224, inhibition of AP-1 signaling by T-5224 and other similar potential agents must assume a high priority for the treatment of fibrotic diseases in well-controlled clinical test settings.

In contrast to the pathways used in the up-regulation of expression of c-Jun by TGF- β and PDGF, activation of c-Jun by phosphorylation proceeds through the activity of c-Jun N-terminal kinase (JNK). The selective JNK inhibitor, CC-930, prevented the phosphorylation of cJun, reduced the stimulatory effects of TGF- β and PDGF on collagen production, prevented collagen accumulation and dermal thickening in bleomycin and Tsk-1 mice experimental fibrotic models, and, highly remarkably, it induced regression of established experimental fibrosis (191). Phase III clinical trials with various inhibitors of JNK for the treatment of rheumatoid arthritis and different types of cancer showed that inhibition of JNK is well tolerated in humans (192). Mice treated with CC-930 did not show any signs of toxicity and initial results from dosing studies in healthy human volunteers have indicated that CC-930 is well tolerated (193). Thus, JNK is a viable target for treatment of fibrotic diseases.

JAK2 and Stat3. Janus kinases (JAKs) are receptor-associated tyrosine kinases with central roles in cytokine- and growth factor signaling. Upon cytokine binding to the receptor,

JAK kinases become activated and phosphorylate tyrosine residues in the cytoplasmic region of the receptor (194). STATs (Signal Transducer and Activator of Transcription) are latent cytoplasmic transcription factors that are recruited to these phosphorylated receptor sites and become activated by phosphorylation by JAKs of a conserved tyrosine residue near the C-terminus after recruitment to an activated receptor complex. Activated STATs then dimerize and translocate into the nucleus where they activate transcription of several target genes (194). JAK2 is a key-regulator of cytokine signaling and alterations of JAK2 signaling cause profound changes in response to cytokine stimulation. Point mutations in the JAK2 gene, which result in constitutive activation of JAK2, are critical in the pathogenesis of myeloproliferative diseases (194,195) and this critical role has stimulated the development of JAK2 inhibitors, several of which are currently being evaluated in clinical trials with promising results (196).

Increased activation of JAK2 and STAT3 were detected in the skin of SSc patients, which persisted in cultured SSc fibroblasts (197). The selective JAK2 inhibitor, TG 101209, reduced basal collagen synthesis in SSc fibroblasts and prevented the stimulatory effects of TGF β on fibroblasts. Treatment with TG 101209 not only prevented bleomycin-induced fibrosis, but also effectively reduced skin fibrosis in the Tsk-1 mice animal model of tissue fibrosis (197). JAK2 may not only act as a downstream mediator of TGF- β but may also amplify TGF- β signaling by stimulating the expression of TGF- β since incubation with TG 101209 caused a dose-dependent decrease in the mRNA levels of TGF- β in SSc fibroblasts. Furthermore, other studies demonstrated that inhibition of STAT3 and overexpression of suppressor of cytokine signaling 1 (SOCS1) reduced the expression of TGF- β (198,199). Considering that several pharmacological inhibitors of JAK2 are available and appear to be well tolerated, targeting JAK2 for the treatment of fibrotic diseases deserves careful consideration.

Peroxisome proliferator-activated receptor gamma (PPAR γ). PPAR γ is a nuclear receptor originally identified in adipose tissue but is broadly expressed and plays a crucial role in glucose and lipid metabolism. Recent studies have described new functions for PPAR γ in the regulation of connective tissue homeostasis. A decrease in PPAR γ expression accompanied progression of fibrosis in experimental models of lung, liver and kidney fibrosis and was a consistent finding in skin and lung tissues from SSc patients (200). In addition, adiponectin levels, a sensitive and specific index of PPAR γ activity, were reduced in the serum of patients with diffuse cutaneous SSc and correlated inversely with the extent and severity of skin sclerosis (201). Thus, adiponectin might have potential as a biomarker in SSc. Several profibrotic factors such as TGF- β , Wnt proteins and IL-13 suppress PPAR γ activity or expression (202). Treatment of normal fibroblasts with the endogenous PPAR γ prostanoid ligand, 15d-prostaglandin J2, or with the PPAR γ pharmacologic agonist, rosiglitazone, abrogated TGF- β induced fibrotic responses (203-205). In addition, PPAR γ blocks EMT and the differentiation of preadipocytes into fibroblasts (206), a putative novel mechanism of tissue fibrosis. Since rosiglitazone is already used in the treatment of diabetes mellitus, evaluation of it as an anti-fibrotic agent in clinical trials appears warranted.

Inhibition of other profibrotic signaling pathways

Wnt signaling. TGF- β appears to be the major factor activating the canonical Wnt pathway in fibrotic diseases probably mediated largely by a decrease of Dkk-1 and although other molecular mechanisms are possible, the addition of recombinant Dkk-1 blocked the stimulatory effects of TGF- β on the canonical Wnt pathway in fibroblasts (207). Wnt signaling caused the transition of resting fibroblasts into activated myofibroblasts, and increased the production of ECM

components. Dkk-1 is currently being investigated as a potential therapeutic target in other diseases and the cumulative data suggest that inhibition of the canonical Wnt pathway might be an effective approach to target TGF- β signaling in fibrotic diseases

Hedgehog signaling. Abnormal stimulation of Hh signaling might be driven by several factors. Hypoxia at levels similar to those observed in skin of SSc patients induces the expression of Shh (208,209). However, a more likely general scenario is that profibrotic cytokines including TGF- β , PDGF, and Wnt drive Shh overexpression (210-212). It has been shown that Shh stimulated resting fibroblasts to differentiate into myofibroblasts and that overexpression of Shh in mice caused accumulation of collagen and dermal thickening (213). Thus, Hh signaling might play a central role in the pathogenesis of fibrosis. Both Smo-specific siRNA or the small-molecule inhibitor LDE223, a highly selective antagonist of the Hh coreceptor Smo effectively blocked the aberrant activation of the hedgehog pathway while LDE223 prevented bleomycin-induced dermal fibrosis (214). In addition, treatment of Tsk-1 mice with LDE223 prevented progression of fibrosis and induced its regression (214). Several small molecule inhibitors of Smo including LDE223 are currently being evaluated in clinical anti-cancer trials with minimal side effects reported so far. Thus, pharmacologic inhibition of the Hh pathway might be a promising approach to treatment of fibrotic disorders as illustrated in Figure 3.

Notch signaling. Recent studies in SSc skin suggest that infiltrating T cells expressing the Jag-1 ligand might activate Notch signaling in fibroblasts leading to their transition to myofibroblasts with increased expression of ECM (102,103). Although Notch signaling may be induced under hypoxic conditions or by TGF- β , the detailed molecular mechanisms responsible for this transition remain to be determined (215-217). Such activation of Notch signaling has also been found in mouse models of dermal fibrosis (218). Treatment with the γ -secretase inhibitor DAPT,

which blocks Notch signaling, significantly decreased experimental pulmonary and dermal fibrosis in mice and in SSc dermal fibroblasts (102,219). Since various inhibitors of γ -secretase are in clinical trials for different types of malignancies, similar trials in fibrotic diseases appear both feasible and warranted.

Other important pathogenetic mechanisms in fibrotic diseases

Role of micro RNAs in Fibrosis. MicroRNAs (miRNAs) are small (~22 nucleotides), evolutionarily conserved non-coding RNA which modulate the expression of protein coding genes at the post-transcriptional level by binding complementarily to the 3' untranslated region (UTR) of target mRNAs and suppress expression by either inhibiting mRNA translation or facilitating mRNA degradation (220). Approximately 1400 miRNAs have been identified to date in humans with most miRNA genes being located in introns, exons and UTRs of protein coding genes (221). Although the role of miRNA in cancer has been extensively studied, recent interest has been devoted to elucidating their participation in tissue fibrosis and fibrotic diseases (222). Several miRNA, including let-7, miR-21, mir-29 and miR-155 are regulated by TGF- β and their targets are mRNAs coded by genes involved in matrix homeostasis such as those encoding collagens, matrix metalloproteinases, and Smad signaling proteins. miR-29 and let-7 are antifibrotic (223) whereas miR-21 and miR-155 are profibrotic and their expression is increased in fibrotic reactions (224). Conversely, miR-29 was found to be markedly down-regulated in SSc dermal fibroblasts and skin (225). Overexpression of miR-29 in SSc fibroblasts decreased the levels of type I and type III collagens demonstrating a direct effect of miR-29. Similar reduction of miR-29 was observed in fibrotic reactions in other organs, including heart, kidney and lung. TGF- β , PDGF-B or IL-4 reduced the levels of miR-29 in normal fibroblasts to levels similar to

those observed in SSc fibroblasts and a marked reduction was also seen in the bleomycin model of skin fibrosis (225). Although hypothetically regulation of miRNA expression could be therapeutically exploited, presently there are considerable practical barriers to its implementation.

The Immune Response and Fibrotic Reactions. In many instances, a persistent inflammatory reaction characterized by accumulation of immune cells including various lymphocyte populations and macrophages and the release of growth factors and cytokines by these cells lead to activation of myofibroblasts and a fibrotic response. Owing to space limitations and to the availability of several outstanding recent reviews covering the immunologic and inflammatory aspects of tissue fibrosis (226,227), the present discussion will be confined to a brief review of the direct involvement of the immune response in promoting or inhibiting fibrogenesis. Of singular importance in this regard is the role of Toll-like receptors (TLRs) which are activated not only by microbial ligands termed pathogen-associated molecular patterns (PAMPs) which are specifically recognized by TLRs, but also a variety of ligands derived from host components collectively referred to as damage-associated molecular patterns (DAMPs) as well as nucleic acid-containing immune complexes (228). TLRs are found not only in cells of the immune system but also in fibroblasts. Type I interferon production, linked to innate immune signaling through TLRs, can result in TLR3-mediated responses in normal skin fibroblasts (229). While TLR3 is an endosomal receptor, TLR4 is expressed on the fibroblast cell surface and can recognize various DAMPs. Although activation of TLR4 induces a modest fibrotic reaction by itself, it stimulates a synergistic response in association with TGF- β (230). Thus, fibroblast innate immune signaling might be a critical contributor to the perpetuation of a profibrotic response.

The adaptive immune response is complex in that it contributes both profibrotic and antifibrotic aspects. The T_H2 profibrotic responses, are characterized most importantly by the production of IL-13, which has been shown to be a potent stimulator of fibroblast proliferation and collagen production and when overexpressed induced skin and lung fibrosis in various experimental models (231). Elevated levels of IL-13 have been found in patients with SSc and it has been implicated in the pathogenesis of IPF, experimental lung fibrosis, SSc, and liver fibrosis (232-234). A recent study demonstrated that IL-13 signaling via IL-13Ra(2) activated AP1 which then stimulated the transcriptional activity of the TGF- β promoter. In contrast, the inhibition of IL-13 signaling reduced the production of TGF- β and markedly reduced collagen deposition in bleomycin-induced pulmonary fibrosis (235). There are two phase II, double-blind placebo, randomised-controlled trials currently ongoing in SSc-ILD and IPF in which a fully humanized monoclonal antibody against human IL-13 is being tested.

T_H1 effector T cells, as well as natural killer and natural killer T cells, produce IFN- γ which inhibits fibrosis, at least in part, by antagonizing the activity of TGF- β . IFN- γ inhibits the phosphorylation of Smad3 as well as inducing the expression of inhibitory Smad7 (236-238). IFN- γ also blocks the T_H2 cytokine-induced differentiation of peripheral blood monocytes into fibrocytes (239). In spite of this supporting evidence, clinical studies testing the therapeutic potential of IFN- γ in IPF, SSc and other fibrotic disorders have been mostly disappointing (240).

IL-17A, expressed by T_H17 cells, has been implicated in the pathogenesis of pulmonary, cardiac and hepatic fibrosis (241-243). The proinflammatory cytokines IL-1 β and IL-23 have been identified as initiators of T_H17 responses and it has been found that in some circumstances TGF- β may partially exert its fibrotic effects through IL-17A production (241,244). Extracellular

bacteria and certain fungi can lead to inflammasome activation and IL-6 production, which in the presence of TGF- β 1, can drive T_H17 differentiation (226). IL-6 has been reported to be up-regulated in the serum, dermal fibroblasts and peripheral blood mononuclear cells of SSc patients, and found to correlate with the extent of skin fibrosis (245). Blocking of IL-6 *in vitro* decreased collagen production, and IL-6 deficiency attenuated lung fibrosis in the bleomycin model (246). Tocilizumab, a humanized monoclonal antibody directed against the IL-6 receptor, has been approved for the treatment of RA. Although preliminary data in a small number of patients from one study on the effects of tocilizumab on polyarthritis in SSc patients did not show any change in the extent of cutaneous involvement over a 6-month period (247), there is a currently ongoing trial to examine the effects of tocilizumab in diffuse SSc.

Role of Reactive Oxygen Species (ROS) in Fibrotic Diseases

Numerous studies have provided compelling evidence for the involvement of ROS in the development of tissue fibrosis (248-250). Although ROS are produced under physiological conditions and are essential for many intracellular reactions including fibroblast proliferation, there is evidence that SSc fibroblasts produce ROS constitutively and that elevated ROS levels may be involved in the increased collagen expression in these cells (251). In addition increased urinary 8-oxodG levels in SSc patients suggest marked oxidative stress (252). Although several classes of enzymes can produce ROS, in the present context the most important is the NADPH oxidase (NOX) family of oxidoreductases, especially NOX4 which is the most widely distributed isoform. NOX4 is one of seven NADPH isoforms and like the others its structure consists mainly of a six-transmembrane domain known as the gp91phox domain (253,254). Despite their extensive similarity in structure and enzymatic function, members of the NOX family differ in their mechanism of activation. In particular, NOX4 requires interaction with a second

membrane-bound subunit, p22 phox, and in contrast with other members of the NOX family does not require other subunits (255). NOX4 generates predominantly hydrogen peroxide (H₂O₂) rather than superoxide (O₂⁻) (256,257), although it is able to generate superoxide under specific conditions.

Because of the increasing interest in the potential role of ROS in fibrogenic processes in a variety of tissues and the realization that NOX family members are key effectors, substantial efforts are now being devoted to develop selective NOX inhibitors. The first of these to be obtained are the triazolo pyrimidines such as VAS2870 and VAS3947, which inhibit NOX specifically but are not isotype selective (258). These compounds have been shown to be effective *in vitro* in several cell systems expressing different NOX isotypes. However, because of the lack of isotype specificity, intense efforts have been devoted to the development of NOX4-specific or selective inhibitors (259). To date the best of these are pyrazolopyridine dione derivatives, the most potent of which is GKT136901. This and similar compounds demonstrated good oral bioavailability and safety profiles as well as dual inhibitory activity for NOX4 and NOX1 in the nanomolar range and have been shown to inhibit EMT and prevent bleomycin-induced pulmonary fibrosis. These findings suggest that the best pyrazolopyridine dione derivatives warrant clinical trials for treatment of IPF and other fibrotic disorders.

Matrix Stiffness and Fibrogenesis. Recent studies have convincingly demonstrated that the mechanical properties of the ECM can modulate the behavior of resident cells including regulation of their proliferation, biosynthetic activities and stage of differentiation, although the molecular mechanisms mediating these activities are only beginning to be understood. When lung fibroblasts were cultured on polyacrylamide substrates of varying stiffness, they responded by producing increasing amounts of polymerized α -SMA (260,261). This actin polymerization

resulted in nuclear translocation of MKL1, a cofactor that plays a central role in expression of fibrotic genes including α -SMA. Furthermore matrix stiffening promoted production and activation of RhoA, increased ROCK activity and enhanced fibroblast contractility. Inhibition of RhoA/ROCK blocked these responses including MKL1 nuclear translocation and myofibroblast differentiation. Similar results were obtained with hepatic stellate cells which became progressively myofibroblastic with increasing substrate stiffness (262).

These experiments suggest that as fibrogenesis proceeds and tissues become increasingly stiff, a vicious cycle can be initiated in which the stiffness itself can promote further ECM production. Furthermore, these findings suggest that therapeutic approaches aimed at the reduction of tissue stiffness may prove to be extremely potent antifibrotic agents. As discussed above, RhoA/ROCK inhibitors including statins are already available and may be more generally useful as anti-fibrotic drugs than currently understood. A substantial portion of ECM stiffness can be attributed to the crosslinking of collagen mediated by the copper-requiring enzyme lysyl oxidase, rendering controlled inhibition of lysyl oxidase a potential therapeutic approach. In this regard it should also be pointed out that the drug d-penicillamine, which was extensively utilized as an antifibrotic agent in the past (263-266) but was discredited following a high dose versus low dose comparative study (267), is a potent inhibitor of collagen crosslinking and may, therefore, be extremely effective in reducing tissue stiffness. These considerations should lead to the re-evaluation of d-penicillamine as an antifibrotic agent. Another potential approach to the fibrotic stiffness problem in carefully selected cases is the direct injection of clostridial collagenase into the affected tissue. This technique has been used successfully in nonsurgical treatment of Dupuytren's contracture (268).

Inhibition of Fibrosis by an Endostatin-1 Derived peptide. A recent novel and potentially ground-breaking study has shown that a cell-permeable peptide corresponding to the carboxy terminal end of the endostatin-1 molecule is capable of exerting remarkable effects on the pattern of gene expression of human normal fibroblasts in culture (269). The peptide caused a potent antifibrotic effect modulating several aspects of fibroblast biology including a decrease in expression of the important profibrotic transcription factor EGR-1, reduction of the expression of type I collagen and fibronectin, and inhibition of the expression of lysyl oxidase, the main collagen crosslinking enzyme. This study opens up the possibility of utilizing cell-permeable peptides for the treatment of SSc, IPF, and other fibrotic diseases.

Discussion and Conclusions

The present challenges facing the field of fibrotic diseases in terms of therapy bear some similarities to the approach for cancer treatment. The treating physicians are confronted with complex pathogenetic processes which, although similar in their final outcome, are quite diverse in specific molecular mechanisms. Thus, identifying critical targets and appropriate, effective therapies is extremely difficult. Owing to the diversity and heterogeneity of the fibrotic diseases with respect to their etiology and pathogenesis, novel screening approaches will be required to characterize and identify particular pathways operative in individual cases. Substantial progress in this direction has been made in the cancer field, mainly due to the availability of large data bases documenting genetic alterations in malignant cells. While this approach has at best a limited place in fibrotic disease, RNA microarray screening of samples derived from affected tissues is feasible and is currently being implemented. Such analyses have been carried out comparing samples from fibrotic tissue from SSc and IPF patients and control tissues in which

substantial differences were observed between the normal and fibrotic samples. However, based upon this kind of data it has not been possible, as yet, to develop pathogenetic models which could lead to rational therapeutic approaches. As detailed in the text, there has been an explosive growth in new findings documenting alterations in potentially profibrotic growth factors/cytokines and in their signaling pathways. The challenge is how to apply these findings in individual cases. Presently, the only way to determine such changes is by invasive biopsies and even then sophisticated screening and molecular analyses of the samples would be difficult at best or marginally informative. Thus, there is an urgent need for the identification of novel biomarkers, most desirably from serum or plasma samples, which have the power to categorize fibrotic reactions with respect to the active pathogenetic mechanisms and pathways.

A second major difficulty is the lack of suitable surrogate parameters capable of measuring the effectiveness of novel therapeutic agents. As listed in Table I (and this list is by no means complete) there is a plethora of drugs which are attractive candidates based largely on *in vitro* evidence from laboratory studies and supportive animal experimental findings, although they have not been appropriately tested in controlled clinical studies. Constructing well designed controlled clinical trials will be a difficult although a quite necessary step that will require the cooperation of multiple investigators and centers using both non-invasive and invasive measurements as discussed recently for SSc clinical trials (270). On the positive side, many existing drugs have been approved for other purposes (e.g. imatinib, rosiglitazone) eliminating the necessity for extensive safety testing. Another feature that is now becoming apparent and that fibrotic diseases share with cancer is the necessity of using multiple drugs to affect different pathways. This is due to the fact that the pathogenetic mechanisms of fibrotic diseases consist of

complex networks of multiple and often redundant pathways and blocking a single node, while occasionally effective, will usually not be sufficient or effective.

However, it is expected that in the near future translational medicine will be able to successfully overcome these serious challenges and convert the extensive knowledge acquired recently about the cellular and molecular mechanisms of tissue fibrosis into effective therapeutic approaches for the devastating and currently incurable fibrotic diseases.

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FIGURE LEGENDS

Figure 1. Cellular origins and pathways leading to activated myofibroblasts. Insults caused by a variety of putative causative agents such as bacteria, toxins, ROS, or radiation in genetically predisposed hosts result in inflammation. Activated inflammatory cells secrete cytokines and growth factors such as TGF- β which cause fibroblast activation and differentiation of endothelial and epithelial cells into myofibroblasts. These cells produce excess amounts of ECM resulting in dysfunctional fibrotic tissue.

Figure 2. TGF- β signaling pathways critical for the fibrotic response. Illustrated are canonical and non-canonical pathways originating from two representative dimeric receptors. Following TGF- β binding, the TGF β RII receptor recruits a TGF β RI, either activin-like kinase-1 or activin-like kinase-5 and activates it by phosphorylation. Activin-like kinase 5 (Alk 5) then specifically phosphorylates receptor-regulated Smad2 and Smad3 which then complex with Co-Smad4 resulting in their transport to the nucleus where they interact with various co-activators or co-repressors to regulate transcription of critical genes, here represented by connective tissue growth factor (CTGF) and α 1(I) and α 2(I) collagen genes. Also illustrated are several non-canonical pathways. An important one involves the phosphorylation and activation of the cellular Abelson non-receptor kinase (c-Abl) resulting in activation of several downstream critical factors including Smad1, early growth response protein (EgR) and protein kinase C delta (PKC- δ), all of which contribute to the fibrotic response.

Figure 3. Growth factor/cytokine signaling pathways important in fibrogenesis. Illustrated are the signaling pathways for platelet derived growth factor (PDGF), Notch, Wnt and Hedgehog. Each of these pathways is activated by ligand-binding to specific receptors, but the

subsequent signaling transmission mechanisms between these pathways differ dramatically (see text). For clarity of presentation they have been abbreviated with only the essential features presented.

TABLE 1. ANTI-FIBROTIC THERAPEUTIC TARGETS AND PHARMACOLOGIC AGENTS

<u>Target</u>	<u>Agent</u>
TGF- β expression and activation	pirfenidone $\alpha\text{v}\beta 6$ antibody ATI and ATII receptor blockers ACE inhibitors CAT-192 (anti-TGF- $\beta 1$ monoclonal AB) Caveolin scaffolding domain (CSD)
TGF- β Signaling pathways	
Canonical	
TBR1	SM305
SMAD3	SIS3
Non-canonical	
c-Abl	imatinib mesylate
PDGFR	dasatinib
c-kit	nilotinib
PKC- δ	small specific polypeptide rottlerin derivatives
CTGF	monoclonal CTGF antibodies
Inhibition of fibrocyte homing	
CXCL12	antibody
CXCR4	AMD3100 and others
CCR2	inhibitors (PF-04136309)
src	dasatinib, SU6656
VEGFR	nintedanib (BIBF1120)
FGFR	sorafenib
Other intracellular pathways	
JAK2	TG 101209
Wnt	Dkk-1
Hedgehog	LDE223
Notch	DAPT

RhoA

statins,
GGT inhibitor
Y27632, fasudil
caveolin scaffolding domain (CSD)
CC-930

ROCK

MEK, ERK, JNK, Akt,
JNK

Transcription factors

AP-1

Fli-1

Sp1

PPAR γ

T-5524

macrolide antibiotics

intercalating agents

rosiglitazone

Other mechanisms

IL-13

IL-6 receptor

TLR

Nox4 (ROS)

ET-1

Matrix stiffness, collagen crosslinking

humanized monoclonal AB

Tocilizumab

TLR inhibitors (E5564, TAK-242)

GKT136901

Bosentan, other ET receptor blockers

d-penicillamine, Clostridial collagenase

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