Getting Out of a Sticky Situation: Targeting the Myofibroblast in Scleroderma

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Abstract: There is no treatment for the fibrosis observed in scleroderma (systemic sclerosis, SSc). Although genome-wide expression profiling has suggested that differences in gene expression patters between non-lesional and lesional skin are minimal, phenotypically these areas of tissue are quite different. In fact, lesional areas of scleroderma patients can be distinguished by the presence of a differentiated form of fibroblast, termed the myofibroblast. This cell type expresses the highly contractile protein α–smooth muscle actin (α–SMA). Fibroblasts isolated from SSc lesions excessively synthesize, adhere to and contract extracellular matrix (ECM) and display activated adhesive signaling pathways. Strategies aimed at blocking myofibroblast differentiation, persistence and activity are therefore likely to be useful in alleviating the fibrosis in scleroderma.

Keywords: PDGF, TGFβ, endothelin, rac, PPARγ, Smad1, pericyte, egr-1, CCN2, CTGF, PKCε.

INTRODUCTION

Fibrosis has been hypothesized to arise due to a hyperactive tissue repair program. In healthy, uninjured connective tissue, fibroblasts are 'stress-shielded' by the surrounding extracellular matrix (ECM) [1, 2]. Upon tissue damage, fibroblasts, no longer stress-shielded by the ECM, develop progressively stronger attachments to the ECM. These attachments are visualized on cell surfaces in structures called focal adhesions (FAs), which contain clusters of specialized ECM receptors called integrins [1,2]. Subsequently, fibroblasts migrate into the wound and, due to stress and growth factors released by immune and blood cells, differentiate into myofibroblasts. Myofibroblasts, characterized by the expression of the highly contractile protein α–smooth muscle actin (α–SMA) which is organized into stress fibers connected to the ECM [2], produce and remodel new ECM. Although myofibroblasts disappear from newly formed connective tissue during normal repair, myofibroblasts persist in fibrotic connective tissue, and are considered to be the final effecter cell in all fibrotic conditions including scleroderma [systemic sclerosis, SSc, 3]. Indeed, although genome-wide expression profiling has failed to identify differences in mRNA expression between clinically affected and unaffected (i.e. fibrotic and non-fibrotic) areas of SSc skin [4], histological and phenotypic explorations have revealed that clinically affected, but not clinically unaffected, areas of SSc patients are abundantly populated by myofibroblasts [3,5]. Moreover, fibroblasts isolated from clinically affected, but not unaffected, areas of SSc patients are characterized by elevated adhesion to and contraction of ECM. For all these parameters, fibroblasts taken from healthy skin behave similarly to fibroblasts taken from non-lesional areas of SSc patients.

The underlying mechanistic difference among clinically affected and unaffected skin (and healthy tissue) is unclear. The myofibroblasts could, in principle, emerge due to differentiation of resident fibroblasts in response to cytokines or due to recruitment of α–SMA expressing cells from elsewhere, notably the vasculature (either pericytes or circulating bone marrow cells, also termed fibrocytes) [6,7]. These concepts have been reviewed elsewhere [6, 7]. In this review, I wish to focus on what is known about SSc fibroblasts and how these observations might fit into a conceptual framework of how the myofibroblast might originate in fibrosis.

TRANSFORMING GROWTH FACTOR-β (TGF-β)

ALK5/Smad3

That TGFβ, which consists of three isoforms (TGFβ–1,-2 and -3), is essential for experimentally-induced fibrosis is well-established [8, 9]. TGF-β target genes include ECM components such as collagen and fibronectin and also key markers and effectors of myofibroblast differentiation including α–SMA and the matricellular protein connective tissue growth factor (CTGF, CCN2). The basic canonical TGFβ signaling pathway involves that ability of active TGFβ ligand to signal through a heteromeric receptor complex consisting of a TGFβ type I and a TGFβ type II receptor. Fibroblasts or epithelial expression of the TGFβ type II receptor has recently shown to be necessary for the development of experimental lung fibrosis [10, 11]. In the context of fibroblasts, the TGFβ type I receptor is termed activin linked kinase 5 (ALK5) which phosphorylates Smad2 and 3. Phosphorylated Smad2 and 3 bind to Smad4, and the resultant complex translocates into the nucleus to activate transcription by binding to the sequence CAGA. Smads are relatively weak transcriptional activators, but recruit to the promoter transcriptional cofactors such as p300 [12].

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TGFβ has multiple functions such as suppressing the immune system and epithelial proliferation; thus, targeting TGFβ is likely to result in substantial clinical side effects [8, 9]. A clinical trial evaluating an anti-TGFβ1 antibody was recently conducted for SSc. Results obtained during this trial illustrated that the antibody was ineffective and caused serious adverse effects which may or may not be linked to the use of the antibody [13]. Moreover, targeting ALK5 using small molecule inhibitors reverses some aspects of lesional dermal scleroderma fibroblasts, but critically does not reduce α-SMA or CCN2 protein overexpression or α–SMA stress fiber formation characteristic of this cell type [3, 14]. In addition, CCN2 overexpression in SSc fibroblasts does not involve Smad3 [15]. Finally, SSc fibroblasts overexpress endoglin which suppresses canonical TGFβ signaling [16]. Thus canonical TGFβ signaling contributes to some, but not all, of the phenotype of lesional SSc fibroblasts [3, 14]. That said, however, SSc fibroblasts are in an environment of elevated TGFβ (coming from immune cells) and exogenous TGFβ can further activate gene expression in SSc fibroblasts thus, *in vivo*, canonical TGFβ signaling is likely to be important of the overall fibrotic phenotype in SSc [17-20].

Given these above concerns, it is likely that it is worthwhile to examine efforts expended the precise mechanism through which TGFβ causes its fibrogenic effects. Indeed, substantial evidence now links non-canonical TGFβ signaling pathways to the fibrosis in SSc.

ALK1/Endoglin/Smad 1

Recent data suggest that TGFβ is able to activate Smad1 through ALK1/endoglin (intriguingly a pathway normally considered to operate principally within the vascular system) and that this pathway contributes to the overexpression of profibrotic genes in SSc by elevating ERK [21]. Endoglin, however, appears to be refractory toward canonical TGFβ signaling *via* Smad3 [16]. Increased levels of endoglin correlated with high levels of pSmad1, collagen, and connective tissue growth factor (CCN2); depletion of endoglin resulted in reduced expression of these markers [22]. Moreover, this non-canonical ALK1/endoglin pathway may contribute to the overexpression of endothelin-1 (ET-1) by SSc fibroblasts [22]. These results are particularly intriguing as, as described above, myofibroblasts in fibrosis (including in scleroderma) have been postulated to originate from microvascular pericytes; these sets of observations are supportive of this interesting possibility [6, 23, 24] (please see below). Thus targeting the non-canonical TGFβ ALK1/endoglin/Smad1 pathway may prove in the future to be a viable anti-fibrotic approach in SSc.

Syndecan 4/ERK/ets-1

As discussed above, even though gene expression profiling has identified no clear differences between non lesional and lesional skin, clear phenotypic differences exist between these tissues, in that lesional fibroblasts are myofibroblasts, and, when isolated and cultured, possess α–SMA stress fibers and are highly adhesive and contractile [3]. The proteoglycan syndecan 4, a fibronectin coreceptor, appears to be overexpressed selectively in lesional SSc fibroblasts compared to non-lesional SSc fibroblasts and appears to be essential for nucleation of α–SMA stress fibers and hence for ECM contraction [3, 25]. Thus expression of syndecan 4 may be important for the appearance of the myofibroblast phenotype in lesional SSc skin. Syndecan 4 also appears to be important for both TGFβ and mechanical loading to induce ERK activation [3, 26, 27]. Downstream of TGFβ and ERK, transcription factor ets-1 controls the induction of the CCN2 promoter [28, 29]. The transcription factors fli-1 and ets-1 occupy the same sites on promoters; TGFβ stimulation results in replacement of fli-1 on the CCN2 promoter with ets-1 [30]. Indeed, ets-1 seems to generally regulate the expression of profibrotic genes in fibroblasts [31-33]. Thus, targeting the syndecan4/ERK/ets-1 pathway may be a good target for anti-fibrotic therapy in SSc [33].

Egr-1

Recent studies also implicate the immediate-early response transcription factor early growth response (egr)-1 in the pathogenesis of fibrosis, and, in particular, downstream of TGFβ [34]. TGFβ upregulates egr-1 also by an ERK-dependent Smad-independent mechanism and this contributes to the induction of type I collagen in fibroblasts [35, 36]. Egr-1 knockout mice are resistant to bleomycin-induced skin scleroderma [37]. Intriguingly, genome-wide microarray analysis has revealed that only a minority of TGFβ-responsive genes in fibroblasts are also egr-1 responsive; the "egr-1-regulated gene signature" was most prominent in skin biopsies clustering within the "diffuse-proliferation" intrinsic subsets of SSc biopsies, but some of the genes were also associated with the "inflammatory" subset [38]. These observations suggest that targeting egr-1 expression or activity might be a novel therapeutic strategy to control fibrosis in SSc.

PPARγ

An interesting crosstalk exists between TGFβ and the transcription factor peroxisome proliferator-activated receptor (PPAR)γ, which suppresses TGFβ signaling [39]. Recent *in vivo* evidence has directly linked PPARγ with fibrosis. For example, wild-type mice treated with the PPARγ ligand rosiglitazone are resistant to bleomycin-induced skin fibrosis while fibroblast-specific conditional knockout mice deficient in PPARγ show enhanced susceptibility to bleomycin-induced skin fibrosis [40, 41]. Providing a link with SSc, SSc skin fibroblasts inherently display reduced PPARγ expression, and rosiglitazone reverses the overexpression of fibrogenic markers by SSc skin fibroblasts [42]. Although PPARγ agonists or reduction of PPARγ expression have no effect on protein expression of cellular Smad3 or Smad7, Smad-dependent activity is modified through impacting transcriptional coactivator p300 [41, 43]. (It should be pointed out that the PPARγ agonist rosiglitazone is known to have toxic side effects, for example, increasing the risk of myocardial infarction; nonetheless pioglitazone (an agent in the same thiazolidinedione drug class) has not been reported to have this effect [44]. Moreover, it is possible that novel PPARγ agonists not in the thiazolidinedione drug class may be generated in the future.) Whether PPARγ agonists may be useful in the future to treat SSc awaits further study.

CELL ADHESION

Cell Adhesion/Contraction-Mediated Activation of Latent TGFβ

Compared to their non-lesional and healthy counterparts, lesional SSc fibroblasts are characterized by enhanced abilities to adhere to and contract extracellular matrix (ECM) [4]. Adhesion to ECM itself is sufficient to elicit a fibrogenic mRNA expression profile in fibroblasts [45]. In addition, adhesive signaling is elevated in SSc fibroblasts [46-48]. Lesional SSc fibroblasts show an enhanced ability to adhere to ECM in a fashion that is blocked by anti-integrinβ1 antibodies [49]. Mice harboring a deletion for integrin β1 in fibroblasts are resistant to bleomycin-induced skin fibrosis and exhibit delayed tissue repair [50, 51]. These issues arise due to the inability of integrinβ1 deficient fibroblasts to adhere to and contract ECM [51]. They could also arise due to the fact that integrinβ1-deficient mice are defective in activation of latent TGFβ [51].

Integrins activate latent TGF-β1 through two different mechanisms [52]. First, integrins appear to serve as a docking point for both latent TGF-β1 and activating proteases, bringing both into close vicinity enhancing liberation of active TGFβ. Second, integrins appear to engage cell traction forces that are directly transmitted to the latent TGFβ complex resulting in TGFβ release or presentation to its receptors. In normal dermal fibroblasts, expression of integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ is low but is up-regulated in SSc, correlating with transition of fibroblasts into myofibroblasts [53, 54]. Available data indicate that integrins containing the αv subunit bind to and activate latent TGF-β1 through an RGD sequence in the latency associated peptide [55, 56]. In normal fibroblasts, growth factor-induced myofibroblast contraction increases latent TGF-β1 activation in through integrins, in a fashion that is dependent on actin-dependent contraction [51, 57].

Cell Adhesion-Mediated Signaling

In addition to its effect on activating latent TGFβ, cell adhesion to ECM also promotes downstream signaling in response to TGFβ involves the clustering of other proteins in addition to integrins to FAs, including paxillin (which provides a platform for FAK), protein kinase C as well as members of the Rho family of GTPases [58, 59]. FAK/src is required for the ability of TGFβ to induce JNK and TAK1 and hence myofibroblast formation and activity [60, 61]. SSc lung fibroblasts display constitutive ALK5-independent JNK activation, which contributes to the persistence of the myofibroblast phenotype of this cell type [17]. Thus targeting TAK1/JNK may be useful in SSc.

Mice harboring a fibroblast-specific deletion for rac1, a member of the Rho GTPase family, are resistant to bleomycin-induced skin fibrosis [62]. Rac1-deficient fibroblasts are responsive to TGFβ, however they show reduced generation of reactive oxygen species [63]. Restoration with hydrogen peroxide alleviates the Rac1-deficient phenotype both *in vivo* and *in vitro* [64]. Rac is constitutively activated in lesional SSc fibroblasts and pharmacological inhibition of Rac1 reverses the fibrotic phenotype these cells [64]. In this latter system, Rac1 acts by a PI3 kinase/Akt-dependent mechanism; rac inhibition reduced the enhanced Akt phosphorylation observed in SSc fibroblasts [64]. Similarly, TGFβ-induced lung myofibroblast differentiation involves the PI3kinase/Akt cascade [65]. Akt phosphorylation is elevated in SSc fibroblasts [40]; expression of the phosphatase PTEN (which dephosphorylates PI3 kinase and hence suppresses Akt phosphorylation) is decreased in SSc fibroblasts [66]. Loss of PTEN is sufficient to result in skin fibrosis *in vivo*; restoration of PTEN in SSc fibroblasts rescues the profibrotic phenotype of these cells [66].

Of the PKC family members, only protein kinase Cε (PKCε) contains an actin-binding motif [67]. PKCε knockout mice and fibroblasts show defective tissue repair and myofibroblast differentiation both basally and in response to TGFβ [68]. PKCε-deficient fibroblasts show reduced rac activation and the baseline defects observed in the cells can be rescued by rac1 overexpression, indicating that PKCε acts upstream of rac1 [68]. It appears that Akt regulates ROS generation by modulating expression of the NADPH oxidase Nox4 and p22(phox) catalytic subunits, which are both required for NADPH oxidase activity [69]. These data suggest that targeting a PKCε/rac/Akt/PI3 kinase/ROS cascade may useful in SSc.

ADDITIONAL CYTOKINES/MATRICELLULAR PROTEINS

Endothelin

Endothelin has three forms, ET-1, ET-2, and ET-3 [70] that signal through the ET_A and ET_B 7-transmembrane G-protein-coupled receptors [70]. Similar to TGFβ, ET-1 can induce ECM production in fibroblasts, through MEK/ERK and the ET_A and ET_B receptors, whereas ET-1 induces myofibroblast formation, migration and ECM contraction through ET_A and Akt/rac [17, 71]. TGFβ induces ET-1 through JNK, and ET-1 is a downstream mediator of TGFβ's fibrogenic responses [17, 72]. ET overproduction in SSc lung fibroblasts is TAK1/JNK-dependent but ALK5-independent, and contributes to the persistent myofibroblast phenotype of SSc lung fibroblasts [17]. TGFβ works together with ET-1 to promote myofibroblast differentiation [73]. Moreover, gingival fibroblasts (gingivae do not scar) show a less potent response than dermal fibroblasts to a 6 hour treatment with TGFβ (in terms of CCN2, type I collagen and α–SMA mRNA expression) [74]. Gingival fibroblasts do not express ET-1; adding back ET-1 rescues this phenomenon [74]. As discussed above, *in vivo*, SSc fibroblasts are in an environment of excess TGFβ, so that SSc fibroblasts overexpress ET-1 constitutively and that TGFβ and ET-1 synergize in terms of their fibrogenic activity is likely to be of clinical relevance. That SSc fibroblasts are inherently different from normal fibroblasts is emphasized by the fact that they maintain their fibrotic characteristics in culture, show activated adhesive and contractile properties, and overexpress key fibrotic markers independently of canonical TGFβ signaling. Based on all these observations, it is plausible that SSc fibroblasts, due to ET-1 overexpression, are inherently primed to respond excessively to pro-fibrotic signaling emanating from cytokines produced in response to tissue injury or inflammation. Clinical experiments have suggested that ET receptor antagonists may be useful at reversing skin fibrosis

in SSc and IL-2, IL-6, IL-8 and IFN- γ levels in SSc patients but the results have been less positive when SSc-related interstitial lung disease has been examined [75-77].

Platelet-Derived Growth Factor (PDGF)

PDGF, a family of homo- or hetero-dimeric growth factors including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, binds two different PDGF receptors, α and β [78, 79] PDGF stimulates migration and proliferation of neutrophils, macrophages, fibroblasts and smooth muscle cells as well as granulation tissue formation [78, 79]. PGDF, like TGFβ and ET-1, stimulates fibroblasts to contract floating collagen gel matrices, express collagen and migrate [80, 81], and, *in vivo*, for the expression of α–SMA (in the liver) and pericytes to differentiate into myofibroblasts (in the kidney) [82, 83].

Evidence is accumulating that SSc myofibroblasts may arise through the recruitment of microvascular pericytes. PDGFβ receptors are expressed by activated microvascular pericytes in patients with early SSc, but not in those with late-stage scleroderma [84]. Although histological examination of markers is inherently imprecise method of detecting origin, only a subset (~30%) of myofibroblasts in cutaneous mouse wounds are positive for the pericyte marker NG2, but the overwhelming majority of cells in SSc and in bleomycin-induced skin fibrosis are NG2-positive [24, 85]. Imatinib mesylate, which inhibits the platelet-derived growth factor receptor (PDGFR)-beta inhibitor and c-abl, delays wound closure, concomitant with reduced expression of collagen type I and numbers of myofibroblasts [86]. However, *in vitro*, imatinib mesylate did not prevent serum-induced contraction of collagen gels but potently inhibited fibroblast proliferation [86]. In a mouse model of bleomycin-induced dermal fibrosis, dual inhibition of c-abl and PDGF receptor signaling bdasatinib and nilotinib potently reduced the dermal thickness, the number of myofibroblasts, and the collagen content of the skin in a dose-dependent manner at well-tolerated doses [87]. Moreover, it has been shown that TGFβ-induces fibrosis *in vivo via* c-abl [88, 89], in a fashion that appears to involve protein kinase c delta [90]. Collectively, these data suggest that imatinib mesylate may act, at least in part, by blocking pericyte recruitment to SSc skin. In an open label trial, adverse events were common, but improvements in skin thickening were seen [91]. However, in another trial, imatinib was poorly tolerated which could limit its application in SSc [92]. Thus it remains debatable as to whether imatinib mesylate might be a viable option for the treatment of SSc, clinically.

CCN2

CCN2, an excellent surrogate marker for the severity of fibrosis in SSc [93-96], was initially considered to be a classical growth factor but now has been recognized to be a matricellular protein (of the CCN family) that acts through a variety of integrins and HSPGs or trkA to promote cell adhesion and adhesive signaling in response to extracellular ligands [97-99]. CCN2 is induced by both TGFβ and ET-1 [15, 100, 101], and thus may impact the adhesive signaling ability of these cytokines. Indeed, CCN2 promotes the adhesive signaling of TGFβ in fibroblasts [102]. CCN2, independent of canonical TGFβ pathways, is overexpressed in SSc fibroblasts through an ET-1/ the noncanonical Smad1 pathway/Sp1-dependent mechanism [14, 21, 103].

Blocking CCN2 with neutralizing anti-CCN2 antibody or siRNA reduces aspects of bleomycin-induced lung fibrosis including collagen and α–SMA expression [104]. Moreover, fibroblast-specific CCN2 knockout mice are resistant to bleomycin-induced skin fibrosis [105]. CCN2 is not considered to cause fibrosis directly, unless massively overexpressed [106], but rather appears to create an environment favorable for fibrogenic stimuli to act [107]. Rather than being a downstream mediator of TGFβ activity, CCN2 appears to acts as a cofactor with TGFβ to induce fibrogenic activities both *in vitro* and *in vivo* [97, 102, 108]. In cells expressing CCN2 constitutively, CCN2 appears to be required for TGFβ to maximally induce type I collagen and α–SMA [102]. On the other hand, in cells CCN2 not making constitutively, CCN2 is not needed for this process [105, 109]. CCN2 may perform its actions by increasing the bioavailabilty of TGFβ to its receptors at extremely low concentrations of TGFβ [110], or by activating the non-canonical Smad1 pathway [111]. One of the great conundrums that have been revealed by gene expression profiling and by functional analysis of SSc skin and fibroblasts is that SSc fibroblasts taken from clinically unaffected skin already overexpress profibrotic markers such as CCN2 yet, unlike fibroblasts in clinically affected (lesional) skin are not highly contractile myofibroblasts [3,4]. Collectively, these observations suggest that the expression of CCN2 in non-lesional skin fibroblasts may indicate that these cells are inherently primed to excessively respond to profibrotic stimuli (such as TGFβ) hence resulting in clinically defined scar tissue.

FUTURE PROSPECTS AND CONCLUSIONS

Although clinical studies targeting individual cytokines in SSc have been inconclusive, a combinatorial approach may be warranted. Studies examining the signaling mechanisms underlying the action of these cytokines on fibroblasts have revealed that common pathways seem to be used utilized. Blocking these downstream pathways (e.g. c-Abl, TAK, FAK, PPARγ, integrins, Rac/Akt/PKCε) which appear to integrate signaling from growth factors and appear to be essential for the SSc myofibroblast phenotype might prove to be more fruitful. CCN2 may be a good antifibrotic target as it appears to be downstream of all fibrogenic pathways.

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CONFLICT OF INTEREST

Declared none.

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