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Molecular Aspects of Regulation of Collagen Gene Expression in Fibrosis

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Fibrosis, the hyper-accumulation of scar tissue, is characterized by the overproduction and deposition of type I and III collagen by fibroblasts and is the one of the main pathologic outcomes of the autoimmune disorder scleroderma. While the causes of fibrosis in scleroderma are unknown, cytokines such as TGF-β, IL-4 and IL-13, play a crucial role in the stimulation of collagen production have been implicated in the disease process. In fibroblasts stimulation of collagen production by these cytokines is dependent on the Smad and STAT6 signaling pathways induced by TGF-β and IL-4, IL-13 respectively. Furthermore, mounting evidence suggest cytokine crosstalk is relevant in the sclerotic process. Our laboratory demonstrated an increase in TGF-β1 gene transcription from fibroblasts stimulated with IL-4. In addition, TSK/+ mice lacking the IL-4α receptor show impaired transcription of the TGF-β1 gene and did not display fibrosis. Likewise, it appears that STAT6 plays a role in fibroblast TGF-β1 transcription after IL-4 or IL-13 stimulation. These findings suggest that an epistatic interaction between IL-4 and TGF-β may exist which is crucial for pathologic sclerotic activity.

KEY WORDS: Fibrosis; scleroderma; pro-fibrogenic cytokines; fibroblasts; collagen.

INTRODUCTION

The extracellular matrix (ECM) is comprised of numerous multifaceted structures that provide structural integrity to the connective tissue. The building blocks of the ECM consist of collagens, glycoproteins (fibronectins, tenascins, fibrillins), glycoaminoglycans and proteoglycans. The ECM acts as a "skeleton" for cells to adhere to grow, maneuver and differentiate. The most abundant proteins in the ECM are members of the collagen family. The collagen protein superfamily consists of 26 genetically distinct proteins. A common feature of the collagen family is to form highly organized polymers (1). For example, the COL1A1 gene generates an element of type I collagen, called the pro-α1(I) chain. This chain combines with another pro-α1(I) chain and also with a pro-α2(I) chain (produced by the COL1A2 gene) to make a molecule of type 1 pro-collagen. These triple-stranded, rope-like pro-collagen molecules are enzymatically processed outside the cell. They then arrange themselves into long, thin fibrils that cross-link to one another in the extracellular space. This results in a collagen mesh which gives connective tissue its tensile strength and elasticity (2–4).

Type I collagen is the most plentiful and well-studied member of the collagen family; it is classified as one of the fibrillar collagens and is one of the most widely expressed proteins in the body being a major constituent of the skin, ligaments, tendons, bone, and numerous interstitial connective tissues, excluding brain, hyaline cartilage and vitreous body. The main function of type I collagen is to provide strength, and elasticity to connective tissue. Other types of collagen are often more restricted in expression profile. For example, type X collagen is exclusively found in hypertrophic cartilage (4).

Normally, the ECM is engaged in a constant flux of remodeling via regulated phases of synthesis and degradation of its components. Fibroblasts are the key players for the generation, deposition, and remodeling of the ECM (5) during development, response to injury and fibrotic disorders. This homeostasis is changed during wound healing when the balance is favored towards an increase in production and deposition of ECM proteins. Fibroblasts are recruited to the wound site by the release of inflammatory mediators, TGF-β and platelet derived growth factor

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(PDGF). There they are stimulated by local growth factor release to produce a ECM protein constituents such as type I and III collagen and fibronectin (5, 6). Proteolytic enzymes such as matrix metalloproteinases, help to degrade collagen in the wounds, and are secreted by macrophages in addition to fibroblasts (7).

Abnormalities in wound healing result in keloids and hypertrophic scars- these are both defined by an excess of accumulation of collagen within the wound. More importantly, increased activity of pro-fibrogenic cytokines such as TGF-β1 and IL-13, have also been implicated in abnormal scar tissue accumulation (8, 9). An excessive deposition of connective tissue components, particularly collagen, is the major pathological finding in fibrotic disorders such as scleroderma, osteogenesis imperfecta, and fibrosis of the lungs, kidneys and liver. In fibrotic disorders, ECM homeostasis is disturbed favoring a situation where a permanent imbalance exists resulting in the replacement of the normal connective tissue architecture with amorphous sclerotic material consisting of type I and III collagen (9).

Cytokines and growth factors are two components that contribute to the metabolic regulation of the expression of collagen genes. The synthesis of collagen in fibroblasts is increased by pro-fibrogenic cytokines including TGF-β1, IL-4, IL-13, IL-6, and PDGF. However, collagen production can be downregulated by pro-inflammatory cytokines such as IFN-γ and TNF-α (10–14). Herein, we will present our current knowledge on the pro-fibrogenic cytokines and the signaling pathways they are involved in the regulation of collagen synthesis and its affects in the fibrotic disorder, systemic scleroderma (SSc) (15).

SSc is a complex, multisystemic, connective tissue disorder believed to be of autoimmune origin characterized by an overproduction of, and local or diffuse deposition of collagen in the skin and some internal organs. Genetic and environmental factors may be involved in the pathogenesis of the disease. Vascular damages, excessive collagen accumulation, and immune activation are commonly identified in patients with SSc.

Vascular injury is best illustrated by the Raynaud's phenomenon, scleroderma renal crisis and pulmonary hypertension. Accumulation of collagen results in fibrosis on the skin and internal organs. Autoimmunity associated with SSc is illustrated by the presence of autoantibodies to DNA topoisomerase I, centromere, RNA polymerase I and III, U3-RNP, NOR-90, PN-Scl and fibrillin-1, in sera of patients (15–17).

The Tight Skin (TSK) mouse represents the most thoroughly investigated animal model of human scleroderma. TSK syndrome is manifested by an increased thickness of the dermis (cutaneous hyperplasia), connective tissue hyperplasia of ventral side of xyfoid cartilage, kidney and adrenal gland (18), pulmonary emphysema (19) and cardiac hypertrophy (20). TSK mice spontaneously produce autoantibodies specific for scleroderma autoantigens such as topoisomerase I, RNA polymerase I, fibrillin-I and nucleoli (16, 21).

In contradistinction with human scleroderma which is a multifactorial disease, TSK syndrome is due to a tandem, in frame, duplication of exons 17–40 of the Fibrillin-1 (Fbn-1) gene (22). The TSK Fbn-1 gene displays several mutations in exons 1–17 and 4 replacing mutations in duplicated exons when compared to the Fbn-1 gene of other murine strains (23). It was shown that mutated Fbn-1 is synthesized and secreted by COS cells transfected with TSK Fbn-1 gene or fibroblasts from transgenic mice bearing a high copy number of mutated Fbn-1 gene (24, 25). Expression of the TSK Fbn-1 gene in mouse embryonic fibroblasts increases deposition of type I collagen and of matrix associated glycoprotein (MAGP)-2 in the ECM. This finding suggests that mutated Fbn-1 plays an important role in skin fibrosis in TSK mice since MAGP-2 was increased in the dermis of TSK mice, and more interestingly in fibrotic SSc skin. Furthermore, fibroblasts over-expressing MAGP-2 were found to express threefold more type I collagen via post-translational effects on protein stabilization suggesting a direct link between mutant Fbn-1, MAGP-2, and increased collagen deposition (26).

MOLECULAR MECHANISMS MEDIATING THE INDUCTION OF FIBROSIS BY PROFIBROGENIC CYTOKINES

There is an abundance of experimental and clinical findings to suggest that TGF-β, IL-4 and IL-13 are profibrogenic cytokines by virtue of their ability to enhance collagen gene expression and deposition of collagen that lead to long lasting-fibrosis in certain diseases.

Transforming Growth Factor-β1 (TGF-β1)

The TGF-β family of cytokines, characterized by 6 conserved cysteine residues, has a wide distribution of expression in terms of cell and tissue type. To date, there are nearly 30 proteins in the TGF-β superfamily. The TGF-β superfamily is one of the important signaling pathways used for normal developmental processes such as tissue patterning, apoptosis, and cell cycle progression. The α-granules of platelets represent a major source of TGF-β, which is also produced by macrophages, and regulatory-T cells (27–29). The prototypical, and best studied, member

of the family is TGF-β1. TGF-β1 possesses diverse functional characteristics depending on the cell type in question. For example, TGF-β1 inhibits T cell and macrophage function and suppresses epithelial cell growth yet it serves as a mitogen for fibroblasts inducing proliferation, migration, and synthesis of a host of cellular products. In particular it has been found to be a potent inducer of ECM protein production. As such, TGF-β1 has been implicated as a key cytokine involved in mechanisms of pathogenic fibrosis (30, 31).

TGF-β1 itself is secreted complexed to latent associated peptide (LAP). TGF-β1 and LAP are synthesized as a single polypeptide which is cleaved by furin to generate the non-covalently associated TGF-β1/LAP termed the small latent complex (SLC). Upon secretion the SLC becomes disulfide linked to latent TGF-β1 binding protein-1 (LTBP1) to form the large latent complex (LLC). The LLC is fixed to the ECM via interactions if the C-terminal region of LTBP1 with the N-terminal region of the Fbn-1 component of the microfibrils. Thus, the ECM acts as a reservoir of TGF-β1 activity (29–30).

The TGF-β receptor family is also quite diverse consisting of 7 type I and 5 type II serine/threonine kinase receptors (30, 31). Receptor complexes are heterotetrameric in nature and consist of two type II receptors (TGF-βRII) (75–85 kDa), which can bind TGF-β directly and two signal transducing type I receptors (TGF-βRI) (50–60 kDa) which only interact with the bound TGF-β/TGF-βRII complex (30–32). The TGF-β receptors are glycoproteins of about 500 amino acid residues that contain small cysteine-rich extracellular domains. The type I receptor has a single hydrophobic transmembrane domain and a C-terminal cytoplasmic glycine/serine region (GS domain) that precedes the receptor kinase domain (33). Binding of TGF-β to the TGF-βRII is believed to induce conformational change in the TGF-β exposing binding sites which recruits the TGF-βRI to form a heterotetrameric receptor complex. TGF-βRII by virtue of its intrinsic kinase activity, phosphorylates TGF-βRI on multiple residues in the GS domain leading to a conformational change which provides a binding site for Smad 2 and 3. This action further increases the specificity of the kinase domain for the C-terminal serine residues of its intracellular downstream effectors, and allows for the subsequent phosphorylation of the Smad proteins (31, 34, 35).

The intracellular signaling pathway of TGF-β was illuminated in studies carried out in Drosophila and C. elegans (36, 37). While there are eight currently identified members of the Smad family in humans, only the receptor-activated (R)-Smads 2 and 3 are phosphorylated and activated by the TGF-β receptor complex. When the TGF-β ligand is bound by the receptor, the R-Smads bind to areas in and around the phosphorylated GS region of the TGF-βRI and are subsequently phosphorylated on a C-terminal SSXS motif. Phosphorylated Smad2 and Smad3 form a dimer and further bind to Smad4 (a co-activator Smad) to then translocate into the nucleus. Therefore, Smads in the heteromeric form enter the nucleus to activate transcription of selective genes. Inside the nucleus, the heteromeric Smad complex may bind directly to a CAGAC sequence called the minimal Smad binding element (SBE). However, the Smad interaction with the SBE is weak ($\approx 10^{-7}$ M). Thus, it is only by association with binding partners that Smads can achieve high affinity protein/DNA interactions. To this end, the Smads have been shown to interact with numerous transcription factors including ATF-2, Sp1, GATA-3, AP1, TFE3 and FAST-1 (34, 38–40). Smad3 assists in activating transcription by using co-activators p300 and cAMP response element binding protein (CBP) (39). The cellular level of Smads is controlled by proteolysis, a process in which Smurfs (Smad ubiquitin regulatory factor) plays an important role (41).

Another group of Smad proteins are known as the inhibitory Smads (I-Smad); these include Smad6 or Smad7. These inhibitory Smads prevent R-Smad phosphorylation and further translocation of the heterocomplex consisting of Smad2, Smad3, and Smad4 to the nucleus (42).

Stimulation of fibroblasts with TGF-β1 has been found to rapidly induce a sustained increase in transcription from the type I pro-collagen and fibronectin genes (43, 44). This has lead to the hypothesis that TGF-β may play a central role in the development of pathologic tissue fibrosis (45). In particular scleroderma fibroblasts have been shown to express elevated levels of the TGF-βRI and TGF-βRII mRNA (46). Furthermore, the increased expression of TGF-βR mRNA correlates well with elevated levels of collagenα 2 type I (COL1A2) mRNA levels in the fibroblasts. It is thought that the altered phenotype of the SSc fibroblast is due to over-expression of TGF-βRI and II leading to enhanced autocrine synthesis of TGF-β (46). Supporting this notion, blockade of endogenous TGF-β signaling with antibodies or anti-sense TGF-β oligonucleotides can prevent the over-expression of collagen genes in SSc fibroblasts (47). Likewise, elevated phosphorylation levels of Smad3 and an increased nuclear localization of Smad2 and 3 in SSc fibroblasts suggest chronic autocrine TGF-β-mediated activation (48).

Animal models such as chronic graft versus host disease (cGVHD) and TSK mice have also shed insight into the importance of the relationship between TGF-β signaling and over-synthesis and deposition of collagen in skin fibrosis. Murine cGVHD is characterized by fibrosis, particularly in the skin and lungs (49).

McCormik *et al*. (50) demonstrated that the administration of anti-TGF-β antibodies one and three days after the injection of allogeneic bone marrow cells can prevent the occurrence of scleroderma-like skin and lung alterations.

We have studied the effects of halofuginone on the TGF-β induced collagen synthesis by TSK fibroblasts as well as on the development of TSK syndrome after administration of this drug soon after birth. Halofuginone, is a isoquinoline alkaloid that was shown to reduce skin tensile strength in chickens in addition to inhibiting the synthesis of type I collagen in human fibroblasts (51). It was demonstrated by our laboratory that halofuginone inhibits type I collagen production by obstructing the TGF-β signaling pathway. More specifically, *in vitro* treatment of TSK fibroblasts with halofuginone reduced the amount of synthesis of collagen as consequence of a reduction in the promoter activity and transcription of the collagen gene. The collagen inhibitory activity was due to prevention of Smad3 phosphorylation, its nuclear translocation and its' binding to the Smad3 consensus site, as assessed by EMSA (52). This is in agreement with data showing that Smad3 $-/-$ fibroblasts have reduced profibrotic responses (reduced levels of collagen α1 type I (COL1A1) mRNA expression) when stimulated with TGF-β (53).

Furthermore, the treatment of fibroblasts with halofuginone enhanced the phosphorylation of c-Jun paralleled with enhanced DNA binding and transcriptional activity of the AP-1 transcription factor composed of c-Jun and Fos, inhibition of collagen promoter activity and a reduction in the level of COL1A2 transcript (54). In addition, introduction of a dominant negative c-Jun construct completely ablated the effect of halofuginone on collagen synthesis. C-Jun acts as an antagonist of COL1A2 promoter activity interacting with a negative regulatory sequence located in the -804 to -675 bp region of mouse α2(I) collagen promoter that is suggested to negatively regulate gene activity in TSK mouse fibroblasts (55).

The inhibitory effect on collagen synthesis was confirmed with *in vivo* studies in TSK mice. Upon administering halofuginone to newborn or adult TSK mice, a decrease in collagenous material was seen, as well as in the number of fibroblasts carrying the transcript of the COL1A2 gene (54, 56). These data taken together demonstrate the crucial role of TGF-β in fibrosis of human diseases such SSc and in corresponding animal models. The signaling pathway initiated by TGF-β leading to the enhanced expression of collagen gene, is under a tight control of the activation of R-Smads, in addition to activation of inhibitory Smads, whereas degradation is controlled by the Smurf gene.

IL-4 and IL-13

In addition to TGF-β, other pro-fibrogenic cytokines such IL-4 and IL-13 stimulate fibroblast collagen production by different signaling mechanisms and are involved in the development of the fibrotic disorders such as scleroderma.

IL-4 is a pleotropic cytokine that is produced mostly by Th2 T cells, NK1 cells and mast cells (57–59). IL-4 has been shown to have approximately 30% homology with cytokine IL-13 (60). However, while they have several overlapping biological activities they exhibit distinct functionalities. For instance, IL-4 can drive differentiation of T cells towards the Th2 polarity and serves as a B cell mitogen, while IL-13 is the primary cytokine implicated in murine models of granulomatous disease and allergic lung inflammation (61, 62).

Both IL-4 and IL-13 work through the IL-4 receptor (IL-4R) or IL-13 receptor (IL-13R) respectively. Lymphocytes incubated with IL-4 show that the binding to IL-4/IL-13 receptor complex initiates a Janus-activated kinase/STAT (JAK-STAT) signaling cascade, which results in the activation of STAT6 (63). The human IL-4R is a heterodimer complex that is composed of the IL-4Rα chain and γc chain while the IL-13 receptor complex is made of 3 different components: the IL-4Rα chain, the low-affinity binding chain IL-13Rα1, and the high-affinity binding chain IL-13Rα2 (64). Therefore, receptors for both cytokines share the IL-4Rαchain, and support STAT6 activation (65). The α-chain of IL-4R is associated with JAK1 and the γc chain of IL-4R$\alpha$is connected with JAK2. IL-13Rα1 is associated with Tyk2. These signaling factors are imperative for STAT6 activation. JAK1 and STAT6 are activated by IL-13, however JAK2 is not. These studies suggest that STAT6 activation may be due to STAT6 binding to the to the IL-4Rα chain (65).

In human fibroblast cell lines, IL-4 and IL-13 stimulation can both induce phosphorylation of JAK2, Tyk2, STAT6 and IRS-1 proteins (66). Therefore, it may be suggested that signal transduction via the IL-4 and IL-13 receptor in the fibroblast cell lines is similar and may be one explanation for the apparent redundant effects of the cytokines in fibrosis pathogenesis (66).

IL-4 has been demonstrated to play a role in collagen production from fibroblasts. Stimulation of collagen using IL-4 was first seen in 1992, using fibroblastic synovial cell lines from patients with rheumatoid arthritis and osteoarthritis (14). It was also determined that IL-4-stimulated fibroblasts expressed higher levels of procollagen α1 type 1 transcripts in comparison to controls. Based on these preliminary observations, it was concluded that IL-4 had an effect on collagen production at the

pre-translational level (14). Lee and colleagues performed studies to verify whether or not IL-4 could play a role in transcriptional regulation of collagen gene expression in SSc fibroblasts. They demonstrated that IL-4 could be important in promoting biogenesis of collagen proteins in addition to increasing stability and transcription of collagen mRNA (67). Methods such as immunocytochemistry and *in situ* hybridization, have also been used to determine the effects of IL-4 on collagen production. It was shown that antibody staining for IL-4 was significantly enhanced in fibroblasts taken from scleroderma patients whereas control fibroblasts were found to be negative for IL-4 (12). More interestingly, serum IL-4 levels were found to be significantly higher in patients with SSc compared to normal individuals (11, 68).

IL-4 is one important factor in the development of cutaneous fibrosis within *in vivo* mouse models. Our lab has documented that in TSK/+ IL-4Rα−/− or TSK/− IL-4−/− mice, cutaneous hyperplasia is not detected when compared with TSK/+ mice (69, 70). In addition, when antibodies towards IL-4 are administered to young TSK/+ mice, this treatment can prevent the development of dermal fibrosis (71). Ubiquitous over-expression of IL-4 in the skin of transgenic mice show cases focal deposition of collagen and alters the homeostasis of the skin (72).

Recently, we investigated the mechanism of up-regulation of collagen gene expression by IL-4 in normal and TSK fibroblasts. Our results have shown that fibrotic fibroblasts express a much higher level of IL-4Rα and thusly exhibit a much stronger activation of STAT6 upon stimulation with IL-4 compared to wild type fibroblasts, correlating with increased collagen promoter activity and COL1A1 message levels. We also found that introduction of a dominant negative STAT6 construct abrogated this effect, suggesting a critical role for STAT6 in IL-4 induced up-regulation of collagen synthesis (73). This may be due to a direct effect of STAT6 on collagen promoter activity, which is supported by recent data.

In vitro studies indicate that in addition to IL-4, IL-13 can also stimulate collagen production in human fibroblasts (14, 74). Studies in both normal and keloid fibroblasts have demonstrated that IL-13 is necessary for collagen production (9). Furthermore, IL-13 is involved in the up-regulation of collagen α2(I) expression at the transcriptional level. Similar to IL-4, the STAT6 and phosphoinositide-3-kinase (PI3K) signaling pathways are required for transcription of the COL1A2 gene and type 1 collagen expression (74). Therefore, both IL-4 and IL-13 have common signaling characteristics and both are involved in the enhancement of collagen in both murine and human fibroblasts.

EFFECTS OF IL-4 AND IL-13 ON EXPRESSION OF THE TGF-β1 GENE IN FIBROBLASTS

Studies performed on the differentiation of T cells clearly show that cytokines in exogenous milieu may influence the program of cytokine gene expression in various T cell subsets. For instance, it was demonstrated that IL-4 is a necessary differentiation factor for TGF-β Th3 regulatory cells (28) and that IL-4-mediated development of these cells from naïve CD4 T cells take place through a STAT6-independent mechanism (75). It is important to note that TGF-β exhibits opposite effects on lymphocytes and fibroblasts; TGF-β displays anti-inflammatory and immunosupressive functions on the immune system whereas its role in fibroblasts is to stimulate the production of ECM proteins.

The role of TGF-β and IL-4 on the development of skin fibrosis in TSK mice is supported by our data that targeted mutations in the IL-4Rα and in one TGF-β1 allele prevents the occurrence of cutaneous hyperplasia, and lowers the levels of dermal hydroxyproline and serum concentrations of autoantibodies (70). The finding that deletion of either cytokine completely prevented fibrosis suggested that the two molecules must work in consort to generate the sclerotic phenotype as otherwise deletion of either cytokine alone would have a partial effect at best.

This hypothesis was supported by our data demonstrating that IL-4 stimulation of fibroblasts increased TGF-β1 mRNA levels and that TSK/+, IL-4−/− mice had a 80% reduction in TGF-β1 mRNA compared to TSK/+, IL-4+/+ mice (69) (Fig. 1). Therefore, IL-4 appears to regulate the expression of TGF-β in fibroblasts, providing an explanation for the absence of cutaneous hyperplasia in TSK/+, IL-4Rα-/- and TSK/+, TGFβ ± mice.

As we discussed above, the activation of collagen genes in fibroblasts is dependent of Smad-induced signaling pathway or STAT6-induced signaling pathway by TGF-β and IL-4 respectively. In the case of CD4 Th2 cells, IL-4 activates other transcription factors such as GATA3, c-Maf, NF-1, Sp-1 (76). Surprisingly, an inspection of sequence of TGF-β1 promoter showed that it bears not only Smad3 and AP-1 sites transcription factors activated through TGF-β signaling pathways but also STAT6, GATA3, NF-1, Sp1 activated by IL-4 and IL-13 (Table I).

Through Western blotting, we have found that that STAT6, AP-1 and SP-1 are constitutively activated in 3T3, C57BL/6 and TSK fibroblasts (73) and the transcripts of GATA3 and cMaf genes can be detected by RT-PCR (Fig. 2). The presence of the GATA3 transcript in fibroblasts was surprising since it was considered that GATA3 is solely expressed in hematopoietic cells (77). Because of

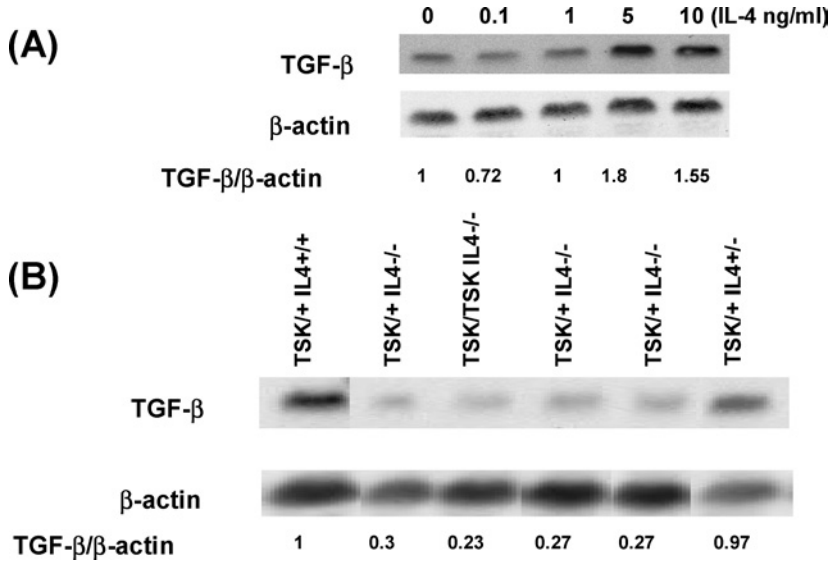

Fig. 1. Northern blotting detection for TGF-β1 in murine fibroblasts. (A) Northern blotting analysis of TGF-β1 transcript in C57BL/6 fibroblasts stimulated with various concentrations of murine recombinant IL-4 (ng/ml). (B) Northern blotting of TGF-β1 transcript in lungs. RNA extracted from the lungs of three TSK/+ and one TSK/TSK mice, bearing a targeted mutation of IL-4. Two control mice are also used (TSK/+ IL-4+/+ and TSK IL-4±). Composite picture adapted from (70).

these observations, we hypothesize a plausible crosstalk network at the level of the IL-4 and TGF-β1 genes. To address this question, we used several deletion constructs of the TGF-β1 promoter containing various combinations of these elements.

Table I. DNA Transcription Factor Binding Sites on the Murine TGF-β1 Promoter

Transcription Factors	Binding Site Location (s)
RREB1	−1785/−1772
	−1523/−1510
	−257/−244
	−92/−79
AP1	−1402/−1394
Smad3	−1282/−1275
GATA3	−1160/−1151
NF1	−1019/−1002
IRF2	−392/−380
	−348/−336
STAT6	−300/−286
p54	−53/−44
SP-1	−165/−159
	−153/−147
	−125/−113
	−69/−57
	−18/−6
	+9/+21

Note. Binding sites on the murine TGF-b1 promoter sequence was analyzed by program MatInspector (82).

Using the 5′ deletion TGF-β1 promoter-CAT fusion construct (−406/+55), we assessed the capacity of transcription factors synthesized by quiescent or IL-4 and IL-13-stimulated fibroblasts able to increase TGF-β1 promoter activity. The data in Fig. 3 shows that the three constructs (−2/+20 bp, −113/+55 bp, −231/+20 bp) containing the Sp-1 binding sites exhibits minimal CAT activity in the basal state or after stimulation with either IL-4 or IL-13. This suggests that the TGF-β1 promoter activation does not involve Sp-1 to any large extent in spite the fact that Sp-1 was demonstrated by EMSA to be activated by IL-4 stimulation in normal murine and scleroderma fibroblasts (73). In contrast, the TGF-β1 promoter constructs containing the STAT6 binding element (−406/+55 bp and −1799/+55 bp) exhibited significant CAT activity in the basal state, which was enhanced approximately 2 fold by IL-4 and IL-13. Lack of effect of TGF-β1 on promoter activity of the −406/+55 (−406 to +55 bp) construct may be explained by the absence of AP-1 and Smad3 binding motif. The −1799 to +55 bp construct containing AP-1, Smad3, NF-1 and GATA3 binding sites in addition to the STAT6 binding site, exhibits higher promoter activity than the −406/+55 construct in basal state and after stimulation with IL-4, IL-13 and TGF-β. The significant increase in promoter activity of this construct after stimulation with TGF-β may be explained by the presence of the AP-1 and Smad3 binding

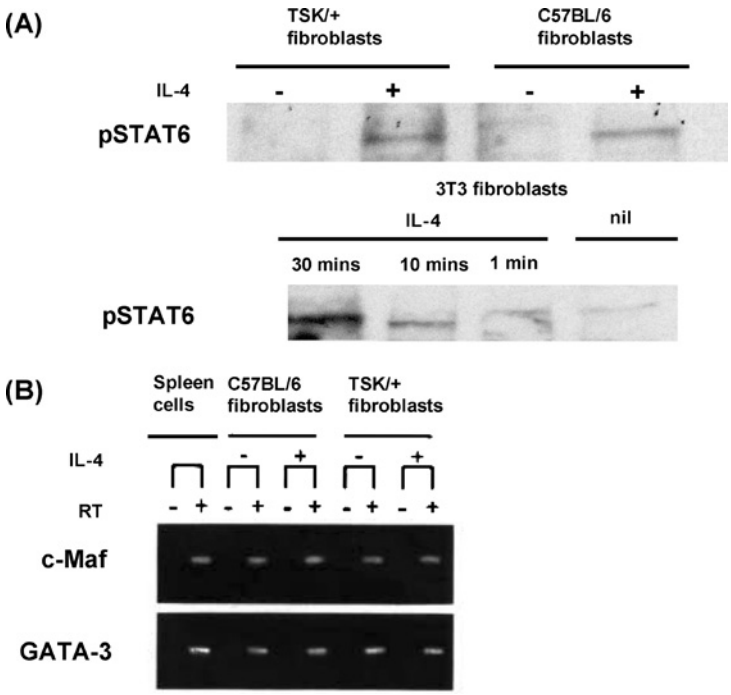

Fig. 2. Western blotting of constitutively active STAT6 and RT-PCR of GATA3 and c-Maf in murine fibroblasts. (A) Western blotting analysis of constitutively activated STAT6 (pSTAT6, Mol Wt 110 kDa) in murine C57BL/6, TSK/+ and 3T3 fibroblasts stimulated with murine recombinant IL-4 (100 ng/ml) or IL-13 (150 ng/ml). (B) RT-PCR analysis of c-Maf and GATA-3 genes in murine C57BL/6 and TSK/+ fibroblasts. Spleen cells are shown as control cells containing both transcripts. R + or − represents the presence of reverse transcription in the sample analyzed.

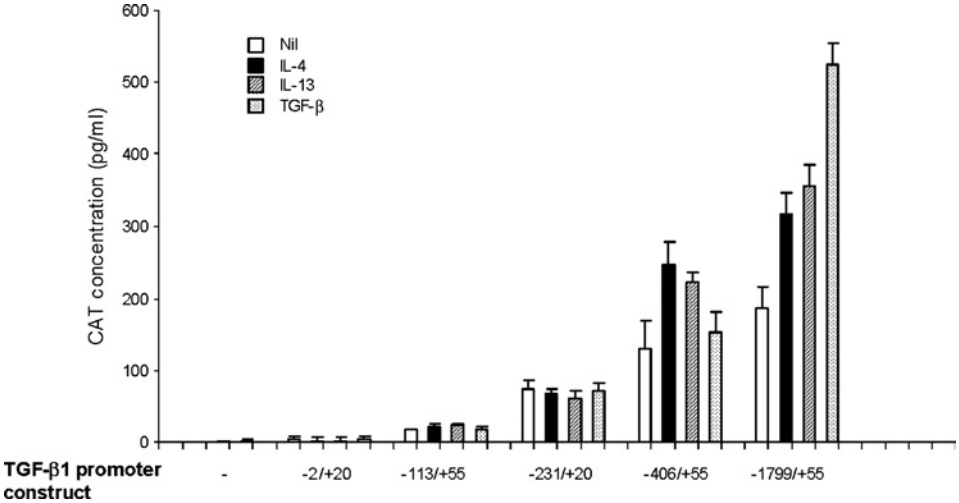

Fig. 3. Effects of profibrogenic cytokines on murine fibroblasts transfected with various TGF-β1 constructs. Murine 3T3 fibroblasts were transfected (using Fugene 6, Roche) with either 1 μg of empty plasmid or with 1 μg of five different TGF-β1 promoter/CAT constructs (regions of −2/+20, −113/+55, −231/+20, −406/+55, −1799/+55). CAT concentrations were measured in fibroblasts cultured for 48 h in medium alone or in the presence of murine recombinant IL-4 (100 ng/ml), IL-13 (150 ng/ml) or TGF-β (10 ng/ml). CAT concentration was measured in cell lysates using the CAT ELISA kit (Roche). Experiments were repeated three times and expressed as the average ± standard error of the means.

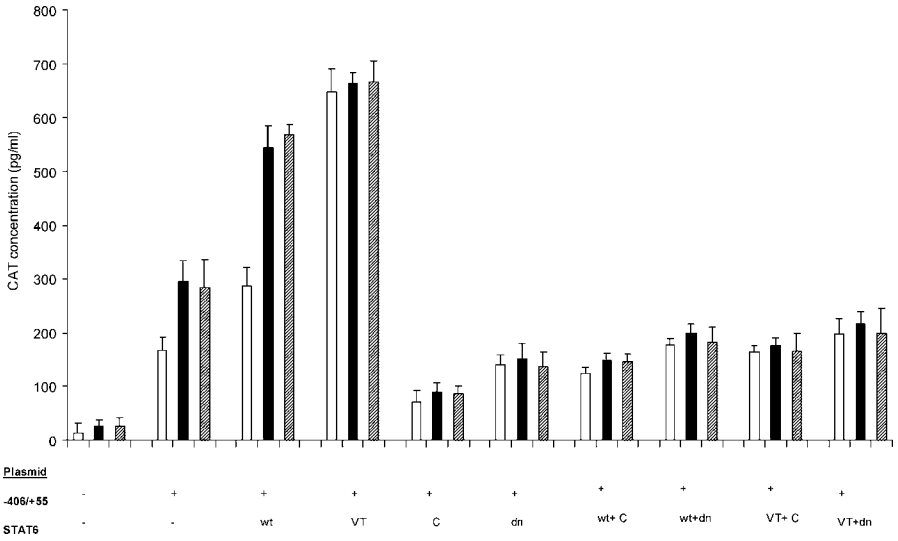

Fig. 4. Effect of IL-4-stimulated and IL-13-stimulated murine fibroblasts. Murine 3T3 fibroblasts were transfected with 1 μg empty plasmid, with 1 μg of the construct containing the -406 to $+55$ base pair (bp) fragment of the TGF-β1 promoter/CAT construct alone, or co-transfected with 0.7 μg STAT6 wild type (wt), 0.7 μg STAT6 constitutively activated (VT), 0.7 μg STAT6 dominant negative (dn), 0.7 μg STAT6 mutant (ΔC) plasmid. CAT concentration was measured in fibroblasts cultured for 48 h in medium alone or in the presence of murine recombinant IL-4 (100 ng/ml) or IL-13 (150 ng/ml). CAT concentration was measured in cell lysates using the CAT ELISA kit (Roche). Experiments were performed three times and expressed as the average $\pm$ standard error of the means.

element in addition to GATA3, NF-1 and STAT6 motifs interacting with corresponding transcription factors known to be activated by IL-4 and IL-13.

To further substantiate the role of STAT6 in enhancing TGF-β1 promoter activity in fibroblasts we studied the activity of the -406 to $+55$ TGF-β1 promoter construct alone or in conjunction with various STAT6 constructs. These constructs included STAT6 wild type (wt), constitutively activated STAT6 (VT) or two dominant negative STAT6 (dn or ΔC). The promoter activity was measured in quiescent murine fibroblasts or upon stimulation for 48 h with IL-4 or IL-13.

Figure 4 shows that stimulation with IL-4 or IL-13 induces a significant increase in the TGF-β1 promoter activity in fibroblasts transfected with the $-406/+55$ construct, or co-transfected with STAT6 wt respectively. While this high promoter activity was observed in fibroblasts co-transfected with the STAT6 VT plasmid, no further increase was seen after stimulation with IL-4 or IL-13. This is in agreement with studies carried out in lymphocytes demonstrating that ectopic expression of STAT6 VT was sufficient to induce maximal activation of STAT6 signaling pathway in Th2 lymphocytes and the addition of IL-4 did not enhance this effect (78).

The ectopic expression of the STAT6 ΔC plasmid strongly inhibited basal activity in fibroblasts transfected with the $-406/+55$ TGF-β1 promoter construct, which was completely resistant to stimulation with IL-4 and IL-13. A significant inhibition in CAT activity was also observed in fibroblasts transfected with STAT6 dn. The difference in the inhibitory effect between the STAT6 dn and STAT6 ΔC constructs is due to the type of deletion; specifically the phosphorylation site in the STAT6 dn construct versus the deletion of 186 C-terminal amino acids in STAT6 ΔC. It is noteworthy that the same pattern of inhibitory effect was observed on promoter activity of the $-409/+55$ TGF-β1 promoter construct in fibroblasts co-transfected with STAT6 wt or STAT6 VT and STAT6 dn or STAT6 ΔC in both resting or IL-4/IL-13 stimulated fibroblasts.

In agreement with data described above, a significant inhibition of promoter activity in both the basal state and after stimulation with IL-4 and separately with IL-13, was observed in fibroblasts transfected with the $-406/+55$ TGF-β1 promoter construct in which the STAT6 binding site was mutated by in-situ mutagenesis (Fig. 5) (Stoica *et al.*; manuscript in preparation).

Since a STAT6-dependent increase of TGF-β1 promoter activity was demonstrated, we further examined if STAT6 has an effect on the transcription of the endogenous TGF-β1 gene. We observed that stimulation of fibroblasts by IL-4 as well as by IL-13 increased TGF-β1

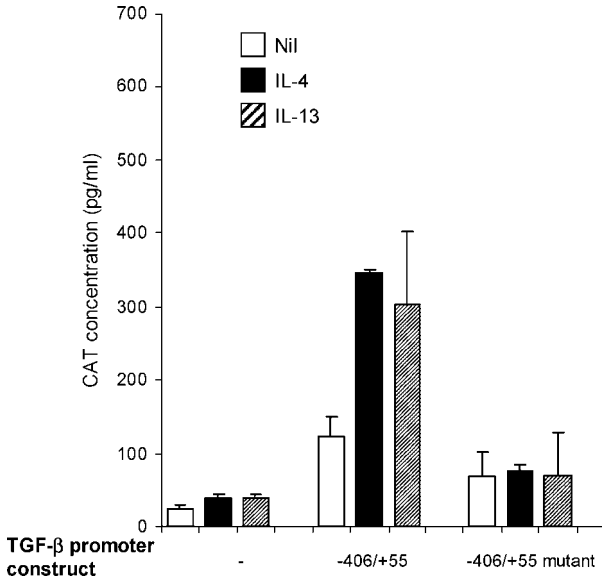

Fig. 5. Effect of mutation of the STAT6 binding motifs on TGF-β1 promoter activity. Murine 3T3 fibroblasts were transfected with 1 μg of empty plasmid or with 1 μg of the -406 to $+55$ bp fragment of the TGF-β1 promoter/CAT construct. In addition, murine 3T3 fibroblasts were also transfected with 1mg of a mutated -406 to $+55$ bp fragment of the TGF-β1 promoter/CAT construct. CAT concentration was measured in the fibroblasts cultured for 48 h in medium alone or in the presence of murine recombinant IL-4 (100 ng/ml) or IL-13 (150 ng/ml). CAT concentration was measured in cell lysates using the CAT ELISA kit (Roche). Experiments were performed three times and expressed as the average $\pm$ standard error of the means.

message levels compared to the basal state. Ectopic expression of STAT6 wt increased TGF-β1 expression in the basal state and this was further enhanced by the addition of IL-4 or IL-13. In sharp contrast, the ectopic expression of STAT6 VT showed a 5.5 increase in basal state, which was not further enhanced upon incubation of fibroblasts with either cytokines (Fig. 6). This is in agreement with studies carried out in lymphocytes demonstrating that ectopic expression of STAT6 VT was sufficient to induce maximal activation of STAT6 pathways in Th2 lymphocytes and the addition of IL-4 did not enhance this effect (78). Additionally, expression of STAT6 dn prevented the up-regulation of TGF-β1 message levels by IL-4 or IL-13. These results corroborate with the promoter activity data described above. These results further support the idea that STAT6 plays an important role in the transcription of TGF-β1 upon IL-4 or IL-13 stimulation. Taken together, these results demonstrate that STAT6 is important in both IL-4 and IL-13 stimulated activity of the TGF-β1 promoter. Figure 7 depicts the mechanisms of the activation of the collagen gene in fibroblasts through signaling pathways initiated by pro-fibrogenic cytokines.

Ongoing studies are aimed at measuring the levels of TGF-β1 transcript and proteins in fibroblasts stimulated or not with cytokines and co-transfected with the $-406/+55$ TGF-β1 promoter construct and wild type or two dominant negative STAT6 plasmids. However, it is possible that IL-4 may induce TGF-β expression via other mechanisms. For example, it was demonstrated that a functional Ras, an upstream element of the mitogen activated protein kinase (MAPK) signaling pathway, is necessary for receptor tyrosine-kinase induced transcription of TGF-β gene (79, 80). As well, it was recently shown that the p38 MAPK pathway is involved in TGF-β gene expression (81).

Our preliminary results suggest a link between the IL-4 and IL-13 signaling pathways and TGF-β production. We identified a STAT6 binding element in the TGF-β1

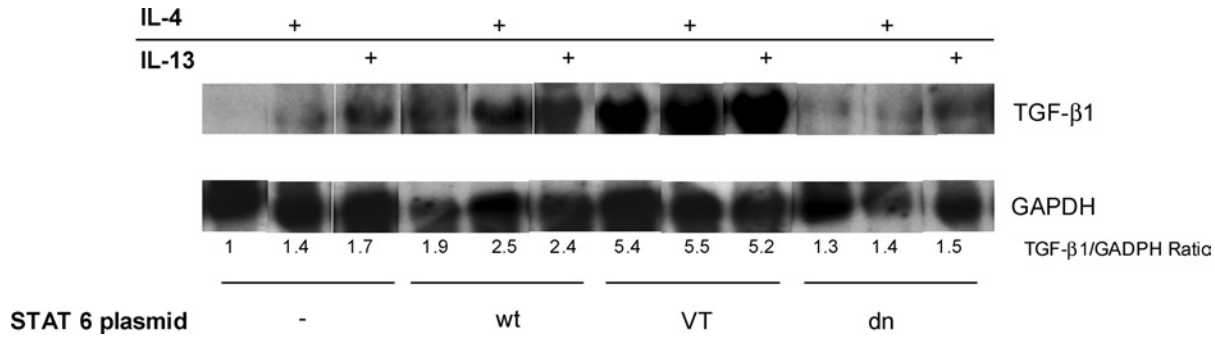

Fig. 6. Northern Blotting of the TGF-β1 and GAPDH transcripts in murine fibroblasts transfected with plasmids containing STAT6 constructs. Murine 3T3 fibroblasts were transfected with empty plasmid or with plasmid containing STAT6 wt, STAT6 VT or STAT6 dn and then cultured for 48 h in medium alone or in the presence of human recombinant IL-4 (100 ng/ml) or IL-13 (150 ng/ml). RNA extracted from transfected fibroblasts were used for determining the level of TGF-β1 and GAPDH transcripts using cDNA probes specific for TGF-β1 and GAPDH. The results were expressed as TGF-β1/GAPDH ratio subsequent to measuring the signal intensity using Adobe Photoshop software. Experiments were repeated twice.

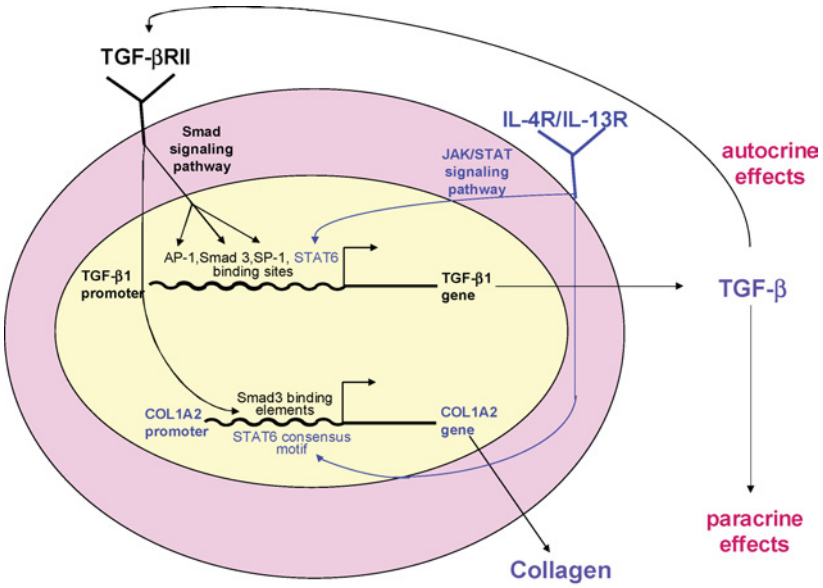

Fig. 7. Activation of the type 1 collagen and TGF-β1 genes in fibroblasts via signaling pathways induced by pro-fibrogenic cytokines. Our laboratory proposes that the pro-fibrogenic cytokines IL-4/IL-13 can regulate the promoter activity of the TGF-β1 gene in addition to the COL1A2 gene, in fibroblasts. As well, TGF-β1 is proposed to possess both autocrine and paracrine effects in regards to the fibroblasts.

promoter and demonstrated that this site is crucial for IL-4 and IL-13 activation of the TGF-β1 promoter, transcription and translation of the TGF-β1 gene in murine fibroblasts. This is the first demonstration of a molecular mechanism of activation of the TGF-β1 gene by pro-fibrogenic cytokines IL-4 and IL-13. These results illustrate the intricate nature of crosstalk in the cytokine network and suggest that molecules interfering with either of the IL-4 or IL-13 signaling pathway in fibroblasts may be a potentially useful therapeutic target in the treatment of fibrotic diseases.

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