MINIREVIEW

Factors Involved in the Regulation of Type I Collagen Gene Expression: Implication in Fibrosis

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Type I collagen, the major component of extracellular matrix in skin and other tissues, is a heterotrimer of two α1 and one α2 collagen polypeptides. The synthesis of both chains is highly regulated by different cytokines at the transcriptional level. Excessive synthesis and deposition of collagen in the dermal region causes thick and hard skin, a clinical manifestation of scleroderma. To better understand the causes of scleroderma or other tissue fibrosis, it is very important to investigate the molecular mechanisms that cause upregulation of the Type I collagen synthesis in these tissues. Several cis-acting regulatory elements and trans-acting protein factors, which are involved in basal as well as cytokine-modulated Type I collagen gene expression, have been identified and characterized. Hypertranscription of Type I collagen in scleroderma skin fibroblasts may be due to abnormal activities of different positive or negative transcription factors in response to different abnormally induced signaling pathways. In this review, I discuss the present day understanding about the involvement of different factors in the regulation of basal as well as cytokine-modulated Type I collagen gene expression and its implication in scleroderma research. [Exp Biol Med Vol. 227(5):301–314, 2002]

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The extracellular spaces in tissues are filled with organized extracellular matrix (ECM), which is composed of proteoglycans like decorin and fibromodulin; fibrous proteins like collagen, elastin, and fibrillin; adhesion molecules like fibronectin and laminin; and different types of matrix metaloproteinases, and play important roles in cell signaling and cellular activities (1). Collagens are the major fibrous proteins in ECM. Different combinations of different α-chains make triple-stranded helical structure of different collagens. To date 19 to 20 types of collagens have been identified. The molecular nature and functions of several collagens have been characterized (reviewied in Ref. 2). Type I collagen, the major component of ECM in skin, bone, ligaments, etc., is composed of glycine- and proline-rich two-α1 (I) and one-α2 (I) chains. α1 (I) and α2 (I) are products of two genes. The pro-COL1A1 and COL1A2 polypeptide chains are synthesized by fibroblasts, osteoblasts, or odontoblasts and enter into endoplasmic reticulum where specific proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine, respectively, which help the pro-α chains to combine with other chains by hydrogen bonds and form the triple helix procollagen structure. Procollagens are secreted by the fibroblasts through the Golgi apparatus in the extracellular space where the N-terminal and C-terminal propeptides are cleaved by specific proteases. The mature processed collagen molecules aggregates to form larger collagen fibrils and help to form the ECM with other components. Therefore, normal structural and functional Type I collagen production and deposition in ECM to make normal physiological connective tissue needs regulation at several steps. Abnormality in any step may cause hypo-, hyper-, or defective synthesis and accumulation of collagen in ECM, which in turn causes different diseases in humans such as osteogenesis imperfecta, scurby, scleroderma or systemic sclerosis, keloids, lung fibrosis, liver fibrosis, etc., (review in Refs. 3–6).

Uncontrolled excessive synthesis and deposition of Type I collagen by fibroblasts in the dermal region of skin increases the thickness of dermal region, which is the hall-

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mark of scleroderma disease (6–9). Therefore, it is very useful to understand the molecular mechanisms that cause the upregulation of Type I collagen gene expression to better understand the scleroderma disease or other collagen-related diseases. To date, several efforts have been made to identify the factors and the signal transduction pathways that are involved in hypertranscription of collagen gene expression in scleroderma skin fibroblasts or in cytokine-treated normal skin fibroblasts. In this review, I summarize the present day understanding about the regulation of basal as well as cytokine-modulated Type I collagen gene expression under normal physiological and pathological conditions and its implication in diseases with special reference to skin fibrosis or scleroderma.

Type I Collagen Genes and Regulatory Elements

Human pro-COL1A1 and pro-COL1A2 genes are located in 17q21.31–22.05 and in 7q21.3–22.1, respectively (10). Human COL1A1 is 18 kb in size and consists of 51 exons (11, 12), and COL1A2 is 38 kb in size and consists of 52 exons (12, 13). Although both subunits of Type I collagen are not linked, they are coordinately regulated to form the functional triple helical Type I collagen protein (14). The ratio of the steady-state levels of pro-mRNA for COL1A1 and COL1A2 is 2:1, which correlates with two COL1A1 and one COL1A2 polypeptides in triple helix structure of collagen (15). Sequence analysis of cloned COL1A1 and COL1A2 structural and regulatory regions show significant homology among species, indicating the collagen genes are evolutionary conserved. Mutations in the structural region have been implicated in connective tissue disorders (reviewed in Ref. 16).

The regulatory regions within the promoter of both subunits contain several repressor and enhancer elements. Several transcription factors interact with these upstream regulatory elements and control the basal (17), cell type-specific (18, 19), and cytokine-modulated Type I collagen gene expression. Cytokine-responsive elements in COL1A1 and COL1A2 genes, like TGF-β response elements, TNF-α response element, and IFN-γ response element have been identified and characterized (reviewed in Ref. 20) and discussed in the "Cytokine modulation of Type I collagen gene expression" section.

The transcription of both COL1A1 and COL1A2 units of Type I collagen is regulated by the promoter as well as first intron (21–24). Several upstream DNA elements as well as first intron have been implicated in skin and lung fibrosis. For example, DNA element between −376 and −108 bp of human COL1A2 is involved in its hypertranscription in scleroderma skin fibroblasts (25). The −376 to +58-bp region of COL1A2 promoter is good enough to drive the activated transcription in SSc lung fibroblasts (26). The region between −174 and −84 bp of human COL1A1 promoter (27) and the region between +380 and +1440 bp corresponding to first intron of human COL1A1 (28) are required for enhanced transcriptional activity and play significant role in the hypertranscription of collagen gene in scleroderma skin fibroblasts.

Like many other genes, DNA methylation of regulatory and structural regions of Type I collagen gene causes its downregulation. Inactivation of gene expression upon DNA methylation may be due to repression of DNA binding of positive transcription factor(s) or to increased binding of MeCPs to methylated cytosine and disruption of specific binding of transcription factor(s) in the regulatory region. Methylation at +7 and +23 nucleotides in COL1A2 first exon causes transcriptional inhibition (29), and increased binding of methylated DNA-binding protein (MDBP) to first exon of COL1A2 gene in response to methylation causes this inhibition (30). Although transformation of human lung fibroblasts by SV40 causes complete inhibition of Type I collagen synthesis and hypermethylation of COL1A1 and COL1A2 genes (31), demethylation by 5-azacytidine has no effect on Type I collagen synthesis (32). Rhodes and Breindl (33) reported that surrounding the start site of murine COL1A1 promoter is methylated in undifferentiated embryonal cells and demethylated in collagen-producing and -nonproducing differentiated cells. Although first intron plays an important role in collagen gene transcription, no region of the first intron is methylated in collagen-producing and nonproducing cells. Interestingly, the first exon is hypermethylated in collagen-nonproducing cells and unmethylated in cells expressing collagen, suggesting other than first exon, first intron and promoter do not play role in methylation-mediated transcriptional control of COL1A1. It will be interesting to study whether elevated Type I collagen gene transcription is at least partly due to undermethylation of COL1A1 and COL1A2 DNA elements in scleroderma skin fibroblasts.

Protein Factors Involved in Type I Collagen Gene Expression

Different well-characterized transcription factors have been shown to interact with promoters and upstream regulatory elements of COL1A1 and COL1A2 genes and are implicated in basal as well as cytokine-modulated Type I collagen gene expression. Activities of some of these factors have been shown to be altered in scleroderma skin fibroblasts. In this section, I summarize the role of different factors in the regulation of Type I collagen gene expression.

CBF. CCAAT binding factor is a heterotrimeric protein with three subunits, CBF-A, CBF-B, and CBF-C (34). All three subunits are required for DNA binding (35), and two transactivation domains are located in the CBF-A and CBF-C (36). CBF binds to mouse COL1A1 and COL1A2 promoters and activates transcription (37). Stable expression of a dominant negative mutant of CBF-B subunit suppresses the mouse fibroblasts growth and inhibits the expression of COL1A2, indicating the potential role of CBF in Type I collagen gene expression (38). Recently, Saitta *et al.* (39) reported that CBF interacts with CCAAT box located at −100 to −96 bp, but not at −125 to −121 bp of human

COL1A1 gene promoter, and CBF binding activity is 3- to 5-fold higher in SSc fibroblasts, indicating its possible involvement in excess collagen synthesis in SSc.

NF1. Nuclear factor-1 is involved in replication as well as transcriptional control of several viral and cellular genes. Rossi *et al.* (40) reported that an NF-1 binding site located in between −350 and −300 bp is required for TGF-β-mediated stimulation of mouse COL1A2 promoter in NIH-3T3 and rat osteosarcoma cells. Point mutations in the NF1 binding site in mouse COL1A2 promoter cause decreased binding of NF1, and inhibition of TGF-β-induced mouse COL1A2 promoter activity further confirms the role of NF1 as a positive transcription factor of COL1A2 (41).

IF1 and IF2. Two different negative factors, IF1 and IF2, and positive factor CBF interact with the COL1A1 gene promoter and control its transcription by down- and upregulation, respectively. IF-1 binds to two upstream control elements located at −190 to −170 bp and −160 to −130 bp of mouse COL1A1 promoter. Mutations in the IF-1 binding sites that inhibit the binding of IF-1 stimulate the COL1A1 promoter activity (42). IF-1 binding site is also present in the mouse COL1A2 promoter in between −165 and −155 bp. Mutation in this region causes inhibition of IF-1 binding and increased COL1A2 promoter activity (14). Presence of IF1 only in collagen-producing cells but not in other cells suggests its role in tissue-specific regulation of the mouse COL1A1 (43). Using highly purified IF2 from lymphocyte nuclear extracts, Karsenty *et al.* (44) demonstrated that IF2 protein binds to mouse COL1A1 promoter and competes with CBF binding. IF-2 interacts with two GC-rich 12-bp repeat sequences located in between −133 and −71 bp. Both the repeats are preceded by CBF interacting CCAAT motif. CBF binding affinity with this element is stronger than IF-2 and, thus, CBF binding inhibits the binding of inhibitor factor IF-2 with the mouse COL1A1 promoter. The 3-bp substitution mutation in the IF-2 binding site that causes decreased binding of IF-2 leads to increased promoter activity, further suggesting the role of IF-2 as a negative regulator of mouse COL1A1 gene transcription. The role of these negative factors in cytokine modulation of Type I collagen gene expression has not been studied.

C/EBP. CCAAT/enhancer binding proteins belong to leucine zipper family protein, which interact with specific DNA sequences present in the regulatory region of many target genes or through protein-protein interaction in the transcriptional initiation complex. At least six members of this family have been identified and characterized: C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and CHOP. Houglum *et al.* (45) reported that C/EBPβ (LAP) binds to mouse COL1A1 promoter, and overexpressed C/EBPβ stimulates the mouse COL1A1 promoter activity in HepG2 cells. Similarly, in primary culture of foreskin fibroblasts, overexpressed C/EBPβ induces the expression of human COL1A2 promoter activity (Ghosh AK and Varga J, unpublished observation). C/EBP-β has also been implicated in acetaldehyde-induced mouse COL1A1 promoter activity in stellate cells where acetaldehyde induces the level of C/EBP-β, which binds to the −365 to −335-bp region of mouse COL1A1 and transactivates (46). C/EBPs interact with Box 5A located in −330 to −303 bp in COL1A2 promoter, and overexpression of a dominant negative form of C/EBP (A-C/EBP) causes the inhibition of TNF-α-mediated repression of COL1A2 activity in NIH3T3 cells, suggesting the potential role of C/EBPs in the TNF-α-mediated inhibition of COL1A2 gene transcription (47). TNF-α inhibits the mouse COL1A1 promoter activity in rat hepatic stellate cells through induction of LIP (p20 C/EBP-β) and C/EBP-δ. Overexpression of LIP and C/EBP-δ mimics the effect of TNF-α (48). All these studies collectively suggest the important role of C/EBPs in basal as well as cytokine modulated Type I collagen gene expression.

Sp1. Sp1 is a zinc-finger family transcription factor that binds to GC-rich consensus sequence and modulates target gene expression. Sp1 binds to three GC-rich regions located between −303 and −271 bp in COL1A2 gene promoter and induces the COL1A2 promoter activity. Introduction of point mutations in the GC boxes abolishes the Sp1 binding and inhibits the COL1A2 promoter activity (49). The Sp1 and Sp1-related Sp3 have been shown to interact with three regions in COL1A2 promoter (−303 to −271 bp, −164 to −159 bp, and −128 to −123 bp) and to be involved in transactivation of COL1A2 promoter in a cell-free transcription system. Introduction of mutation in these three regions revealed that regions −303 to −271 bp and −128 to −123 bp are important in driving Sp1- and Sp3-mediated COL1A2 transactivation (50). Recently, Czuwara-Ladykowska *et al.* (51) demonstrated that Fli-1, a member of ERG subfamily, acts as a repressor of COL1A2 promoter activity, and functional interaction of Fli-1 with Sp1 is essential for this inhibitory action. Fli-1 response element has been mapped between −353 and −186 bp of COL1A2 promoter, which contains Ets and Sp1/Sp3 binding sites (51). The Sp1, Sp3, and CTF/NF1 proteins present in human skin and lung fibroblasts nuclear extracts have been shown to interact with −129 to −107 bp of human COL1A1 promoter. Although overexpressed Sp1 or Sp3 transactivates the COL1A1 promoter, excess Sp3 can block the Sp1-stimulated COL1A1 promoter activity. In TGF-β-treated fibroblasts, the level of Sp3 is decreased, indicating that the altered expression of these Sp1 family proteins under altered physiological conditions may play a key role in regulation of collagen (52). In COL1A1 promoter, two tandem Sp1 binding sites are located in a 274-bp intronic sequence, and DNA-protein complex formed with this element imparts an inhibitory effect on COL1A1 promoter activity (53). It is not clear whether Sp1 or Sp1-related Sp3 interacts with these intronic sequences. The −138 to −77-bp region of murine COL1A1 promoter contains overlapping NF1 and Sp1 binding sites and acts as switch element. Both NF1 and Sp1 stimulate the COL1A1 promoter in Drosophila SL2 cells, and Sp1 is stronger transactivator than NF1. Coex-

pression of NF1 with Sp1 leads to inhibition of Sp1 activation by NF1 (54). Thus, modulation of COL1A1 gene expression depends on the level of a particular transcription factor (NF1 or Sp1) in a particular physiological condition. Interestingly, in normal Ito cells, the Sp1-binding activity is low and significantly induced in activated Ito cells, which is correlated with enhanced COL1A1 gene expression in activated Ito cells. Compared with Sp1-binding activity, the level of NF-1-binding activity is very low in activated Ito cells (55), which may favor the Sp1-mediated transactivation. Similarly, Hitraya *et al.* (27) reported that the region between −129 to −107 bp of COL1A1 promoter interacts with Sp1 and NF1 factors, and Sp1-binding activity in SSc fibroblasts is 3- to 4-fold stronger than normal fibroblasts. Therefore, this Sp1 binding element and its interaction with Sp1 may play a role in SSc development. Increased phosphorylation of Sp1 in scleroderma skin fibroblasts is also associated with increased Type I collagen synthesis, and treatment of SSc fibroblasts with mithramycin, a specific inhibitor of Sp1 binding, abrogates the induced expression of COL1A2 gene (56). Recently, Verrecchia *et al.* (57) also demonstrated that blocking of Sp1 activity using stably transfected antisense Sp1 cDNA causes inhibition of Type I collagen synthesis as well as promoter activities. Therefore, all these evidences strongly suggest the implication of Sp1 and Sp3 along with other factors like Fli-1 and NF-1 in the control of Type I collagen synthesis in normal and SSc skin fibroblasts.

AP1. AP1 is a family of transcription factors that contains JunD, c-Jun, and c-fos, and modulates the target gene expression as homo- or heterodimer. A 29-bp sequence located at the +292 to +670-bp region of first intron of human COL1A1 gene contains an AP1 binding site and involves in transcriptional regulation of COL1A1 gene (58). Ras protooncogene product downregulates the COL1A1 and COL1A2 mRNA synthesis in rodent cells (59), and Ras-mediated inhibition of COL1A1 gene transcription in fibroblasts is achieved through an AP1 site located in the first intron of COL1A1 gene (60). Stuiver *et al.* (61) demonstrated that phorbol 12-myristate 13-acetate (PMA) induces the COL1A2 mRNA and Type I collagen protein levels in PMA responsive mouse 3T3-L1 cell, but not in PMA-nonresponsive 3T3-L1-derived VT-1 cells. Interestingly, in human lung fibroblasts, PMA inhibits basal as well as TGF-β-induced COL1A1 mRNA level in protein kinase c (PKC)-dependent mechanism and possibly through PKC-induced phosphorylation of regulatory proteins (62). The discrepancy of these two observed results may be due to tissue- or cell type-specific differences. As c-jun and c-fos are also activated by PMA, the inhibition of COL1A1 by PMA may be through AP1 response element. Estradiol has also been shown to suppress the Type I collagen synthesis in murine mesangial cells via stimulation of AP1 activity (63). Interestingly, a recent report showed that PMA-induced PKC phosphorylates the MH1 domain of Smad3 (at Ser 37 and Ser 70) and inhibits its direct DNA interaction and its transcriptional activity (64). Yuan and Gambe (65) demonstrated that phosphorylation of serine 89 residue of p300 by PKC represses its transcriptional activity. Therefore, inhibition of Type I collagen synthesis by PMA may be partly due to activation of PKC-induced phosphorylation of p300 or Smad3, which acts as coactivator and activator of TGF-β/Smad-dependent Type I collagen gene transcription (66).

NF-κB. The NF-κB family of transcription factors consists of different subunits such as p65, p50, Rel, and form homo- and heterodimers. In unstimulated cells, NF-κB/Rel family protein dimers generally remain in the cytoplasm as a complex with inhibitor of NF-κB (IκB) protein. In response to external stimuli like TNF-α treatment, IκB becomes phosphorylated by specific protein kinase IKK and undergoes proteosomal degradation. The free NF-κB/Rel transcription factors translocate to the nucleus where they modulate the expression of target genes by direct interaction with the NF-κB binding element or by interaction with other transcription factors or transcriptional coactivator p300/CBP in the transcriptional initiation complex (reviewed in Ref. 67). TNF-α-induced NF-κB translocates to the nucleus and inhibits the COL1A2 promoter activity (68). Overexpression of NF-κB p65 or p50 subunit and c-Rel inhibits the mouse COL1A1 promoter activity in NIH3T3 or hepatic stellate cells, and p65 subunit is stronger inhibitor than p50 or cRel. Interestingly, p65-dependent repression of COL1A1 promoter activity is mediated through Sp1 binding sites located within −220 bp (69).

Smads. Smad family members have been classified into three subgroups based on their structures and functions. TGF-β receptor-dependent R-Smads like Smad2/3 contain N-terminal MH-1 domains, a linker region, and C-terminal MH2 domains where the SSXS motif is known to be phosphorylated and activated by serine-threonine kinase receptors. Co-Smad, Smad4, lacks the SSXS motif. The third group consists of anti-Smad-like Smad6/7, which lacks the MH1 domain and blocks the TGF-β signaling by direct interaction with TGF-β receptor and by blocking the receptor-mediated phosphorylation of R-Smads (reviewed in Ref. 70). MH1 generally helps the Smad to interact with DNA and MH2 domains involved in protein-protein interaction. Transient expression of Smad3 or Smad4 in human skin fibroblasts leads to stimulation of COL1A2 promoter activity. The Smad binding element, CAGACA, located at −263 to −258 bp, interacts with Smads and mediates the Smad-dependent COL1A2 gene transcription. Overexpression of anti-Smad and Smad7 represses the basal as well as TGF-β-stimulated COL1A2 promoter activity, indicating its antagonistic action on TGF-β signaling in human dermal fibroblasts (71). A recent report by Pulaski *et al.* (72) demonstrated that other than modulation of TβR activity and TGF-β signaling, anti-Smad7 may also acts as direct transcriptional regulator like R-Smads. Smad7 acts as repressor or activator of gene transcription based on the nature of promoter, and phosphorylation at Ser249 is important for its transcriptional activity. The authors predicted that Smad7,

possibly through protein-protein interaction, regulates the target gene promoter activity. As in this study, the stimulatory activity of Smad7 has been studied in artificial and mutant construct, and it will be important to find out the activator action of Smad7 on endogenous genes or natural promoters and its modulation by different cytokines like TGF-β, IFN-γ, and TNF-α in a particular cell type.

CBP/p300. The two closely related proteins p300 and CBP are products of two genes and are present in almost every cell type. Although both the proteins share several common structural and functional properties, they have distinct features and, therefore, for many cellular activities, p300 cannot substitute CBP or vice versa. Both proteins interact with several transcription factors as well as receptors and they control the transcriptional status of the target genes of those factors in response to different physiological changes. Therefore, p300/CBP are regarded as adaptor or transcriptional coactivators. The intrinsic HAT activity of p300/CBP modulates the expression of many genes through acetylation of histone and or transcription factors in the transcription complex (reviewed in Refs. 73 and 74). Transcriptional coactivators p300/CBP induce the COL1A2 promoter activity and endogenous Type I collagen gene transcription in human skin fibroblasts. p300/CBP can physically and functionally interact with Smads and mediate the TGF-β-independent and -dependent Type I collagen gene transcription. For Smad-dependent p300-mediated stimulation of COL1A2 promoter activity, complete Smad signaling is required (66). The acetyltransferase activity (AT) of p300 is required for its maximal transcriptional activity in the context of COL1A2 transactivation (66). AT activity involvement in Type I collagen gene expression has also been evidenced from inhibitory effect of HDAC on COL1A2 promoter activity. Overexpressed HDAC1 abrogates the wild-type p300- but not HAT-deleted p300-induced transcription of COL1A2 gene. Sodium butyrate, an inhibitor of HDAC, also stimulates the COL1A2 promoter activity in human skin fibroblasts, further indicating the positive role of HAT in Type I collagen gene expression (Ghosh AK and Varga J, unpublished data).

Myb. Myb belongs to the basic helix-loop-helix leucine zipper family protein and interacts with specific DNA sequences. Piccinini *et al.* (75) reported the increased level of Myb proteins in fibroblasts from SSc. The elevated level of C-Myb mRNA level in SSc fibroblasts has also been demonstrated by Feghali *et al.* (76), and C-Myb is responsible for upregulation of Type I collagen gene in human skin fibroblasts (77). On the other hand, Marhamati and Sonenshein (78) reported that ectopic expression of B-Myb represses the COL1A1 and COL1A2 promoter activity in vascular smooth muscle cells. Involvement of B-Myb in the downregulation of endogenous Type I collagen has been demonstrated further by the same group where basic fibroblast growth factor induces the cellular B-Myb level and leads to the inhibition of the Type I collagen synthesis in vascular smooth muscle cells (79). These data suggest that Myb family protein-mediated modulation of Type I collagen gene expression is cell type dependent.

Myc. The oncogene c-Myc belongs to basic helix-loop-helix leucine zipper protein. Yang *et al.* (80) reported that 3T3-L1 mouse cells, expressing high levels of human c-Myc, suppress Type I collagen mRNA level. c-Myc exerts its inhibitory influence at the transcriptional level. Although c-Myc binds to E-box sequence CACGTG, sequence-specific binding of c-Myc to COL1A1 or COL1A2 promoter has not been characterized. Overexpressed c-Myc cannot block the TGF-β-induced COL1A2 promoter activity in NIH-3T3 cells, suggesting that c-Myc is not a modulator of TGF-β signaling in respect to Type I collagen gene expression. Interestingly, Trojanowska *et al.* (81) reported that scleroderma fibroblasts express increased levels of c-Myc in comparison with control cells. Similarly, Feghali *et al.* (76) demonstrated the elevated levels of c-Myc mRNA in SSc fibroblasts, and these results may be implicated in abnormal growth and activation of scleroderma skin fibroblasts. The direct correlation between induced c-Myc expression and increased Type I collagen production in SSc fibroblasts has not been established.

c-Krox. Zinc-finger family protein c-Krox, a homologue of Drosophila Kruppel factors, is expressed in different tissues. Mouse c-Krox interacts specifically with the G-rich A1 region of mouse COL1A1 promoter and activates the mouse COL1A1 promoter activity in NIH-3T3 cells (82). In contrast to this result, Widom *et al.* (83) demonstrated that human c-Krox binds differentially to human and mouse COL1A1 gene promoters, and overexpressed human c-Krox represses the human, mouse, and rat COL1A1 promoter activity in NIH-3T3 cells. The discrepancy of the results may be due to species-specific functional differences of this factor. Peterkofsky *et al.* (84) demonstrated that unlike G-rich A1-region (−194 to −168 bp) of mouse COL1A1 promoter, analogous COL1A1 promoter region from human (−195 to −168 bp) and rat (−193 to −179 bp) fails to interact with nuclear protein derived from mouse and human cells and possibly is not involved in transcriptional regulation. These results suggest the species-specific involvement of cis-acting regulatory elements and transacting factors in Type I collagen gene expression.

BTEB. Basic transcription element binding protein belongs to zinc-finger family transcription factor that interacts with GC box sequence of different promoter and modulates their transcription (85, 86). In hepatic stellate cells, UV irradiation activates c-Jun N-terminal kinase (JNK) that stimulates the COL1A1 gene expression through a distal GC box located at −1491 to −1470 bp of rat COL1A1 gene. DNA-protein interaction studies revealed that 32-kDa BTEB, but not Sp1, is the interacting protein with this far upstream GC box, and this binding activity is increased in activated stellate cells, suggesting a possible role of BTEB in distal GC box-mediated stimulation of COL1A1 gene transcription in response to UV irradiation and JNK activation (87).

PN-1. Protease nexin 1, a serine protease inhibitor, belongs to serpin superfamily and can inhibit the proteolytic activity of plasmin, thrombin, plasminogen activator, etc. PN-1 has been identified as an upregulated gene in the skin fibroblasts as well as in the dermis of SSc patients using differential display analysis, Northern blot analysis, Western blot analysis, and *in situ* hybridization of skin biopsy from healthy and scleroderma patients (88, 89). As PN-1 has also been detected in non-lesional skin of scleroderma patients, authors suggested that induction of PN-1 might be an early event in the pathogenesis of scleroderma. Overexpression of PN-1 causes stimulation of Type I collagen gene transcription in transient transfection assay, further suggesting its possible involvement in the development of scleroderma (88). PN-1 also acts as inhibitor of ECM degradation (90), indicating PN-1 may increase the level of collagen by increasing the transcription rate as well as decreasing its degradation.

PP1 and PP2A. Overexpressed protein phosphatase 2A but not protein phosphatase 1 activates the human COL1A1 promoter activity in NIH-3T3 cells. Okadaic acid, an inhibitor of phosphoserine- and phosphothreonine-specific protein phosphatase 1 and 2A, inhibits the endogenous Type I collagen synthesis in NIH3T3 cells, indicating that dephosphorylation of some factors is required for activation of Type I collagen synthesis (91). Westermarck *et al.* (92) demonstrated that ocadaic acid inhibits the basal as well as TGF-β-induced Type I collagen synthesis in human skin fibroblasts. Treatment of NIH-3T3 cells with ocadaic acid also inhibits the basal as well as TGF-β-induced human COL1A2 promoter activity. These results suggest that PPI and PP2A positively regulate the Type I collagen gene expression in NIH-3T3 cells, and selective inhibition of PP1 and PP2A may be useful in suppressing the collagen synthesis and tissue fibrosis.

ERK1/2. Extracellular signal-regulated kinase 1/2 represses Type I collagen synthesis in human skin fibroblasts. C2-ceramide, activator of ERK1/2, c-jun N-terminal kinase, and p38 mitogen-activated protein kinase (MAPK), inhibits Type I collagen synthesis. Although C2-ceramide-mediated inhibition can be abrogated by combination of MEK1, MEK2, and p38 inhibitors, only the constitutively active ERK1/2 activator MEK1 but not p38 activator MKK3b can inhibit the Type I collagen synthesis, indicating that ERK1/2 signaling cascades mediate the downregulation of Type I collagen gene expression in fibroblasts in response to mitogenic stimulation (93). On the other hand, Hayashida *et al.* (94) reported that in human mesangial cells, TGF-β1 stimulates the MAPK pathways, and ERK inhibitor or dominant negative ERK1 but not dominant negative mutant SEK (JNK) blocks the TGF-β-induced Type I collagen synthesis, indicating ERK MAPK but not JNK is involved in TGF-β-induced Type I collagen transcription. Dominant negative mutant ERK but not the dominant negative mutant SEK also blocks the TGF-β-induced transcription of 3TP-Lux, indicating crosstalk between ERK and Smads in the regulation of Type I collagen by TGF-β signaling. Therefore, it appears that the effects of ERK1/2 signaling cascades on Type I collagen gene expression is cell type specific (Refs. 93 and 94 and refs. therein).

CBFA1. CBFA1 is a runt-related osteoblast-specific transcription factor involved in osteoblast-specific gene expression in different species. It controls the expression of Type I collagen gene in osteoblasts. CBFA1 binds to the two OSE2 binding sites (at −1347 and −372 bp) in mouse COL1A1 gene promoter and one OSE2 binding site in the first exon (at +12 bp) of mouse COL1A2 promoter, which are both conserved in mouse, rat, and human. CBFA1 activates the COL1A1 and COL1A2 promoter activity via these OSE2 sites (95). Interestingly, TGF-β inhibits the Cbfa1 gene expression, which is regulated by its own product CBFA1. The interaction of TGF-β-induced Smad3 with CBFA1 causes inhibition of CBFA1 activity and Cbfa1 gene expression in osteoblast-like cells. Thus, TGF-β/Smads inhibit the osteoblast differentiation by inhibiting the activity of CBFA1 (96). As both CBFA1 and Smads are involved in the regulation of collagen gene expression, it will be interesting to investigate the role of CBFA1 on TGF-β-mediated COL1A1 and COL1A2 gene expression in osteoblasts.

CTGF. Connective tissue growth factor is a heparin-binding growth factor and plays important role in fibroblast proliferation and wound healing (97). TGF-β stimulates the CTGF synthesis in fibroblasts (98). Like TGF-β, CTGF also stimulates the synthesis of Type I collagen in dermal and lung fibroblasts. Using representational difference analysis, Shi-wen *et al.* (98) demonstrated that the CTGF level is significantly higher in scleroderma skin fibroblasts, indicating that induced synthesis of CTGF may play an important role in fibroblast activation and excessive collagen synthesis.

p53. Overexpressed tumor suppressor protein p53 inhibits basal, TGF-β-induced, and Smad3-induced COL1A2 promoter activity and Type I collagen synthesis in human skin fibroblasts. Treatment of fibroblasts with etoposide, a potent inducer of cellular p53, blocks TGF-β-induced stimulation of COL1A2 promoter activity in a dose-dependent manner. Exogenous p300, a transcriptional coactivator of Type I collagen gene, only partially rescues TGF-β-stimulated COL1A2 promoter activity, and sodium butyrate, an inhibitor of histone deacetylase, blocks p53-mediated inhibition of COL1A2 promoter activity in fibroblasts overexpressing p53. These results strongly indicate that cellular p53 is a potent endogenous suppressor of collagen gene expression in normal fibroblasts. Suppression may involve recruitment of histone deacetylase to the transcription complex by p53. Alterations in p53 expression or function or interaction with other factors may be involved in dysregulated TGF-β responses in scleroderma skin fibroblasts (Ghosh AK and Varga J, unpublished data).

Regulation of Type I Collagen Gene Expression by Cytokines

TGF-β Signaling. The TGF superfamily includes different forms of TGF-β, BMPs, and activins. The TGF-β subfamily consists of β1, β2, and β3 isoforms, which are synthesized as large precursor molecules with pro-peptides. The propeptides are cleaved and mature 25-kDa homodimer remain inactive by latent TGF-β binding protein. Following secretion, the latent TGF-β may interact with membrane-associated receptors like IGF-II or ECM proteins. Different enzymes may participate in the activation of ECM protein-bound latent TGF-β (reviewed in Ref. 99). Different forms of the TGF-β family have different cell type-specific expression and functional involvements. TGF-β induces the synthesis of many ECM proteins such as collagen, fibronectin, laminin, and tenascin, and it inhibits the matrix-degrading enzymes and thus plays an important role in wound healing, fibrosis, embryogenesis, and tumorigenesis (100–102). Active TGF-β binds to TGF-β type II receptor that activates type I receptor by serine phosphorylation. Type I and type II TGF-β receptor form tetramer, and the activated receptors transphosphorylate the Smads in serine residues of C-terminal SSXS motif. The phosphorylated Smads (2 and 3) become active by dissociating the MH2 domain from MHI domain, and they heterodimerize with Smad 4 and translocate to nucleus to activate or modulate the target genes by direct interaction with DNA or by protein-protein interaction in the transcriptional complex (reviewed in Refs. 70, 103, and 104).

Chen *et al.* (71) demonstrated that TGF-β stimulates the human COL1A2 promoter activity through Smad signaling molecules. TGF-β induces the translocation of Smad3 and Smad4 from cytoplasm to nucleus in primary culture of human skin fibroblasts, inhibits the level of Smad3/Smad4 and stimulates the level of anti-Smad, Smad7 suggesting TGF-β activates the Smad signaling in human skin fibroblasts (105). Overexpressed Smad3 stimulates the human COL1A2 promoter activity in absence and presence of TGF-β and the stimulatory effect of TGF-β is mediated through Smad binding CAGACA element located in −263 to −258 bp of COL1A2 promoter (71, 106). The inhibitory Smad of TGF-β signaling pathway, Smad 7 abrogates the TGF-β induced COL1A2 promoter activity (71) further suggesting the involvement of Smad molecules in the control of TGF-β-mediated Type I collagen synthesis in skin fibroblasts. A recent report demonstrated that although Smad2 translocates to the nucleus from the cytoplasm in response to TGF-β in rat cirrhotic liver derived activated hepatic stellate cells (HSC), Smad3 and Smad4 are present in the nucleus in ligand-independent manner and overexpressed anti-Smad, Smad7 cannot inhibit the induced COL1A2 and PAI-1 gene transcription, indicating abnormality in TGF-β/Smad signaling in activated HSC (107).

The transcriptional coactivators p300/CBP participate in the Smad-dependent TGF-β-induced Type I collagen synthesis. p300 stimulates the Type I collagen synthesis, and complete Smad signaling is required for this p300-mediated stimulation as p300 can not stimulate COL1A2 promoter activity in Smad4-deficient MDA-MB-468 breast carcinoma cells (66). The intrinsic HAT activity of p300 is required for p300-mediated maximal induction of basal as well as TGF-β-stimulated COL1A2 transcription in human dermal fibroblasts (66). As recent reports suggested that specific phosphorylation of p300 by PKC and PKB causes the repression of target gene transcription by p300 (65, 108), it will be interesting to study the post-translational modification of p300 in response to TGF-β and its activity in terms of coactivation of Smad-dependent Type I collagen gene transcription.

On the other hand, Chung *et al.* (109) reported that human COL1A2 promoter contains an AP1 binding element in the −265 to −241 bp and binds AP1 but not NF1 or NF-κB, and AP1 binding sequence is essential for mediating the TGF-β-induced Type I collagen gene transcription. Engel *et al.* (110) demonstrated that JNK, a member of MAPK family, is rapidly activated by TGF-β in a Smad-independent manner and induces the AP1 activity as well as Smad3 phosphorylation outside the SSXS motif. JNK-mediated phosphorylation of Smad3 induces the SSXS motif phosphorylation by TβR1 and nuclear accumulation, suggesting a crosstalk between JNK and Smad pathways in TGF-β signaling via AP1. Other reports suggested that TGF-β specifically induces the JunD/AP1 but not c-Jun or c-Fos in human lung fibroblasts, and that JunD is required for TGF-β-stimulated collagen synthesis and 3TP-Lux promoter activation (111).

Besides Smad/p300 and Ap1, Sp1 is also involved in TGF-β-mediated induction of human COL1A2 gene transcription (112, 113). Zhang *et al.* (114) demonstrated that while Smad3/Smad4 complex binds to CAGA box of COL1A2 promoter and is involved in TGF-β-induced transcription, the Sp1 binding site located within the TGF-β response element is required along with Smad binding element for Sp1-Smad functional interaction and full stimulatory effect by TGF-β. Recently, Inagaki *et al.* (115) demonstrated that Sp3 but not Sp1 is the predominant COL1A2 promoter binding activity in parenchymal hepatocytes, which produce very little Type I collagen. While Sp3 overexpression in HSC abrogates the TGF-β-stimulated COL1A2 promoter activity, overexpressed Sp1 stimulates the basal as well as TGF-β response. Sp1 but not Sp3 interacts with Smad3, indicating that cell type-specific interaction of Sp1 or Sp3 with Smads determines the differential expression level of COL1A2 transcription in response to TGF-β in HSC or parenchymal hepatocytes. It is evident from all of these studies that interactions of different factors with Smads are the important criteria for induced Type I collagen synthesis in response to TGF-β, and the involvement of different factors are cell type and species specific.

Positive Modulators of TGF-β Signaling. Several modulators (activators and inhibitors) of TGF-β signaling have been reported in recent years. Here, I discuss in brief the mechanism by which these factors control TGF-β signaling. It will be important to investigate the nature of these modulators in fibrotic tissues to understand whether alteration of the modulator(s) cause(s) abnormal synthesis of ECM proteins like collagen.

SARA. A FYVE domain protein interacts with Smad2 and 3 and recruits them to activated the TGF-β type I receptor that phosphorylates R-Smads. Phospho-R-Smads dissociate from SARA, dimerize with Smad4, and translocate to the nucleus and induce Smad-dependent TGF-β signaling (116). Recently, Goto *et al.* (117) demonstrated that the phosphorylation, complex formation with Smad4, nuclear translocation, and TGF-β-dependent transactivation of a mutant Smad3, which can not interact with SARA, is comparable with those of wild-type Smad3, indicating SARA/Smad3 interaction is not essential for TGF-β/Smad signaling in COS-7 and R-mutant Mv1Lu cells.

TRAP1. TRAP1 interacts with the inactive TGF-β receptor complex and dissociates from the TGF-β-activated receptor complex. Free TRAP1 interacts with Co-Smad, Smad4, and brings it into the receptor-activated Smads to form active smad complex (118).

SPARC. A matricellular protein regulates the ECM production in kidney glomerulus. SPARC appears to be involved in the positive regulation of Type I collagen in kidney mesangial cells possibly through regulation of the level of TGF-β1 (119).

SKIP. Ski-interacting protein SKIP is a nuclear hormone receptor coactivator that interacts with MH2 domain of Smad2 and Smad3 and stimulates the TGF-β-dependent transcription of PAI-1 gene. SKIP overexpression relieves the Ski/Sno-mediated repression, indicating that SKIP is a positive modulator of TGF-β signaling (120).

Negative Modulators of TGF-β Signaling.

Smad7. The anti-Smad, Smad7, blocks the TGF-β signaling pathway by direct interaction with TGF-β receptors and by preventing the receptor-mediated phosphorylation of R-Smads, Smad2 and 3 (121, 122).

TGIF. A DNA binding homeodomain protein recruits HDAC in the TGF-β-induced Smad-containing transcriptional complex and represses Smad-dependent transcription (123). Recently, Pessah *et al.* (124) demonstrated that TGF-β-dependent JNK activation causes inhibition of Smad2 transcriptional activity. Inhibition is due to the enhancement of Smad2-TGIF interaction by activated c-Jun, which in turn causes repression of the interaction of Smad2 with transcriptional coactivator p300.

Smurf. Smurf1 and 2, the E3 ubiquitin ligase containing WW domains, help in protein-protein interaction through PPXY or PY motif of interacting protein. The anti-Smad, Smad7, interacts with Smurfs and translocates to the cytoplasm where Smurfs interact with TβR1 via Smad7. Smurfs induce the ubiquitination of Smad7 and TβR1 and appear to stimulate the inhibitory activity of Smad7 on TGF-β signaling (125, 126).

STRAP. A WD40 repeat protein interacts with TGF-β type I and type II receptors and modulates the TGF-β signaling. Upon interaction with inhibitory Smad7, STRAP stabilizes the interaction of Smad7 with TGF-β receptors and thus helps the Smad7 to strongly inhibit the TGF-β signaling (127).

SNIP1. SNIP1 also suppresses TGF-β signaling by interacting with p300/CBP and preventing the Smad4 from interacting with p300/CBP. Thus, SNIP1 acts as inhibitor of the p300/CBP and TGF-β signal transduction pathway (128).

Ras. Kretzschmar *et al.* (129) reported that oncogenic Ras activates ERK MAP kinases, which phosphorylate the linker region of Smad2 and 3 and inhibit their nuclear accumulation and thus repress TGF-β signaling in epithelial cells.

SnoN. Oncoprotein SnoN represses the TGF-β signal transduction by interacting with Smad2 and Smad4 and suppressing their activities through recruitment of N CoR transcriptional corepressor (130).

Ski. Ski also blocks TGF-β signaling through direct interaction with Smad2, Smad3, and Smad4 and recruiting transcriptional corepressor N-CoR (131).

Lefty. A novel member of TGF-β superfamily, Lefty acts as inhibitor of TGF-β signaling. It inhibits TGF-β signaling through suppression of phosphorylation of Smad2 by activated TGF-β receptor and not through activation of inhibitory Smads like Smad6 or Smad7 (132).

FKBP12. FKBP12 is an immunosuppressant FK506 binding protein that interacts with the cytoplasmic domain of TβR1 (133). Upon ligand-induced phosphorylation of TβR1 by TβR2, FKBP12 is released from TβR1. Inhibition of interaction between TβR1 and FKBP12 by FK506 increases the TGF-β signaling, indicating that FKBP12 is an inhibitor of TGF-β signaling (134). On the other hand, Okadome *et al.* (135) reported that FKBP12 and TβR1 interact *in vivo* in a ligand-independent manner, and the juxtamembrane region of TβR1 is essential for this interaction. Deletion mutation in the FKBP12 interacting region shows the transcriptional responses, and TβR1 does not phosphoylate FKBP12, suggesting that FKBP12 is not necessary for TGF-β signaling; rather, it modulates the TβR1 function.

TRIP-1. A WD domain-containing protein, TRIP-1 associates with the Type II TGF-β receptor in a kinase-dependent way and is phosphorylated by receptor kinase (136). Overexpressed TRIP-1 represses the Smad-, TβR1-, or TGF-β-induced PAI-1 gene promoter activity (137), supporting its negative role in TGF-β/Smad signaling.

IL-1β Signaling. Interleukin-1 (IL-1), a product of monocytes/macrophages, plays an important role in activation of fibroblasts and in synthesis of MMPs, PGE2, different cytokines, and ECM components. Although IL-1β stimulates the synthesis of Type I collagen protein and

mRNA in normal human skin fibroblasts, it has less effect on the skin fibroblasts derived from scleroderma, suggesting the role of this cytokine in the earlier stages of the disease (138). Mauviel *et al.* (139) reported that in human dermal fibroblasts, IL-1β stimulates the Type I collagen mRNA synthesis and inhibits the Type I collagen protein synthesis, suggesting regulation by IL-1β at the post-transcriptional or translational level. Inhibitory effect of IL-1β on Type I collagen in human dermal fibroblasts derived from old donors (>60 years) is greater than dermal fibroblasts derived from young donors (<20 years), suggesting that physiological aging has influence on the degree of responsiveness to IL-1β (140). Goldring *et al.* (141) demonstrated that IL-1β stimulates the Type I collagen protein and mRNA level of COL1A1 and COL1A2 in human chondrocytes when IL-1β-induced PGE2 synthesis is blocked by cyclooxygenase inhibitor indomethacin. Similarly, IL-1β induces the synthesis of Type I collagen in BALBc/3T3 fibroblasts, and this stimulatory activity is greater in indomethacin-treated fibroblasts. Exogenously added prostaglandins inhibit the -222COL1A1 promoter activity. The difference in binding activities of nuclear extracts from control and prostaglandin-treated fibroblasts with the −84 to −29-bp region of COL1A1 promoter suggests that PGE-responsive factors may interact directly or indirectly with basal regulatory elements to control collagen synthesis (142). In Ito cells, IL-1β inhibits the procollagen synthesis and has no effect on procollagen mRNA levels, indicating that IL-1β regulates the Type I collagen synthesis in Ito cells at the post-transcriptional level (143). In human lung fibroblasts, IL-1β stimulates the production of PGE2 and inhibits the production of Type I collagen. Treatment of cells with indomethacin partially represses this inhibition. IL-1β inhibits the transcription rate of COL1A1 gene, indicating that IL-1β suppresses the COL1A1 gene expression at the transcriptional level and repression is PGE2 independent as well as cytokine-induced PGE2 dependent (144). The human recombinant IL-1β stimulates the synthesis of Type I collagen protein and mRNA. Recombinant IL-1β induces the synthesis of PGE2, and inhibition of the PGE2 synthesis by indomethacin does not influence on IL-1β-stimulated collagen production (145). Therefore, it is apparent that IL-1β modulates the Type I collagen synthesis at different levels, and the variations may be due to cell type, species, and age differences. At present, the molecular mechanisms by which IL-1β modulates the Type I collagen gene transcription and its protein synthesis in different cells and under different physiological conditions are not clear.

IFN-γ Signaling. Interferons (IFNs) are synthesized by T-cells, macrophages and by different infected cells. There are two types of interferons, type I (IFN-α and IFN-β) and type II (IFN-γ). IFN-γ binds to the IFN-γ receptor complex (IFNGR1 and IFNGR2) and then receptor associated janus kinase (JAK) becomes activated which in turn phosphorylates and activates the signal transducer and activator of transcription (STAT1α). Once phosphorylated, STAT1α dimerize and translocate to the nucleus where they modulate the target gene transcription either by direct interaction with γ-activated sequences or through protein-protein interaction. (reviewed in Ref. 146). Although IFN-γ represses the basal as well as TGF-β-stimulated Type I collagen gene expression, the STAT1-DNA interaction has not been implicated in these inhibitory actions of IFN-γ (147–151). IFN-γ inhibits the basal as well as TGF-β-induced COL1A1 promoter activity, and the region between −129 and −107 bp of COL1A1 promoter, which contains NF1 and Sp1 binding sites, is sufficient to drive the IFN-γ-mediated inhibition, suggesting that IFN-γ inhibits the Type I collagen synthesis at the transcriptional level. However, mutations in NF1 and Sp1 sites, which abrogates the binding of these factors, repress the basal COL1A1 promoter activity but is unable to abrogate the IFN-γ-mediated inhibition, suggesting that NF1 and Sp1 are not involved in this inhibitory action of IFN-γ (151). IFN-γ also inhibits the basal as well as TGF-β-induced COL1A2 gene expression in human dermal fibroblasts. This inhibition is also at the level of transcription (150, 152). Higashi *et al.* (150) identified a region within −161 to −125 bp of COL1A2 promoter that interacts with specific protein complexes and mediates the IFN-γ-mediated inhibition of COL1A2 promoter activity in human dermal fibroblasts. Further studies are required to understand the exact molecular mechanism governing the IFN-γ mediated inhibition of Type I collagen gene expression.

TNF-α Signaling. The pro-inflammatory cytokine TNF-α is produced by monocytes/macrophages. Trimer TNF-α interacts with two TNF-α receptors (TNFR1 and TNFR2) and activates them, which transmits signals to the nucleus via different transcription factors (153). TNF-α inhibits the Type I collagen mRNA as well as protein synthesis in dermal skin fibroblasts (139). TNF-α represses the collagen promoter activity, indicating regulation at the transcriptional level. TNF-α-responsive element has been colocalized with TGF-β-responsive element in the −378 to −345-bp region of COL1A1 promoter. TNF-α induces the nuclear translocation of LIP, C/EBPβ, and C/EBPδ, and inhibits COL1A1 gene expression (48). In human COL1A2 promoter, TNF-α-responsive element is located at −271 to −235 bp and contains the noncanonical AP1 and NF-κB binding sites. Mutation in the NF-κB site abolishes the TNF-α response. In human dermal fibroblasts, TNF-α stimulates the nuclear translocation of NF-κB, which interacts with the NF-κB binding site and inhibits the COL1A2 promoter activity (68). In a separate report, C/EBP, the leucine zipper family protein, which interacts with CCAAT/ and a consensus DNA element, has been implicated in TNF-α-inhibited COL1A2 promoter activity in NIH-3T3 cells. TNF-α induces the synthesis of C/EBPs and its binding to the −330 to −296-bp region of COL1A2 promoter. Overexpression of a dominant negative form of C/EBP (A-C/EBP) blocks the TNF-α-mediated inhibition of human COL1A2 promoter activity as well as endogenous COL1A2 gene transcription (47). As this dominant negative C/EBP can

inactivate all C/EBPs, it is not clear which form of C/EBP is involved in TNF-α-inhibited COL1A2 transcription. Participation of other forms of C/EBPs in this inhibitory action has also been hinted upon by Greenwel *et al.* (47) as C/EBPβ−/− fibroblasts also respond to TNF-α. Therefore, TNF-α imparts its inhibitory influence on Type I collagen synthesis via different factors and regulatory elements.

Antagonistic Effect of IFN-γ and TNF-α on TGF-β-Induced Type I Collagen Gene Expression

IFN-γ versus TGF-β. While TGF-β upregulates the Type I collagen synthesis in skin fibroblasts, another pleiotropic cytokine, IFN-γ, abrogates this TGF-β-induced Type I collagen synthesis. This conclusion comes from the results on promoter activity, mRNA level, and protein level (147–152). As Smad signaling molecules have been implicated in the TGF-β signaling pathway for Type I collagen gene expression, one can first consider the possible involvement of anti-Smad, Smad 7, in the IFN-γ-mediated repression of TGF-β induction. Ulloa *et al.* (154) reported that IFN-γ abrogates the TGF-β stimulation of TGF-β responsive element containing reporter constructs in fibrosarcoma cells by inducing the level of Smad7. Induction of Smad7 by IFN-γ is JAK-STAT1 dependent. Induced Smad7 interacts with receptors and blocks the receptor-mediated phosphorylation of R-Smads like Smad3 and blocks its nuclear translocation and thus transcription of the target genes. On the other hand, in human primary culture of skin fibroblasts, the mechanism of abrogation of TGF-β-induced Type I collagen synthesis by IFN-γ does not follow this model, where (i) IFN-γ does not induce the mRNA or protein level of anti-Smad, Smad7, (ii) overexpression of antisense Smad7 induces the TGF-β induction but does not block the IFN-γ-mediated inhibition of TGF-β-induced COL1A2 promoter activity in skin fibroblasts, and (iii) IFN-γ does not block the TGF-β-induced translocation of Smad3 and Smad4 in skin fibroblasts. Therefore, the IFN-γ-mediated inhibition of TGF-β-induced Type I collagen synthesis in skin fibroblasts is not mediated through inhibitory Smad7 and thus inhibitory effect may not be at the receptor level (152).

As IFN-γ can block the ligand-independent, Smad3-induced COL1A2 promoter activity, the IFN-γ effect on TGF-β signaling is at the downstream of receptor level or at nuclear level. IFN-γ blocks the TGF-β-induced collagen synthesis through JAK-STAT pathway as, unlike JAK1-expressing U4A cells, IFN-γ can not block the TGF-β-induced COL1A2 promoter activity in JAK1-deficient U4A cells (152), and overexpression of a dominant negative form of STAT1 (TY701) blocks the IFN-γ-mediated inhibition of TGF-β-induced COL1A2 promoter activity in human skin fibroblasts (Ghosh AK, unpublished observation). Overexpression of transcriptional coactivators p300/CBP relieves the IFN-γ-mediated inhibition of TGF-β-stimulated COL1A2 promoter activity, suggesting the functional involvement of p300/CBP in the antagonistic action of IFN-γ and TGF-β where IFN-γ-activated STAT1α sequesters the endogenous p300 and reduces its interaction with Smad3 and thus blocks the TGF-β/Smad-induced collagen gene transcription (152). In a separate report, Eikelberg *et al.* (111) demonstrated that JunD/AP1 is required for TGF-β-mediated stimulation of collagen synthesis as well as for expression of 3TP-Lux reporter gene expression. IFN-γ abrogates the TGF-β-induced collagen synthesis and 3TP-Lux reporter activity in human lung fibroblasts, and STAT1 is required for this IFN-γ-mediated inhibition of collagen synthesis. As expression level of p300/CBP transcriptional coactivators is very low in human lung fibroblasts, and both JunD/Ap1 and Stat1 interact with p300/CBP, the authors proposed that IFN-γ-activated STAT1 and TGF-β-activated AP1 compete for limiting amounts of p300/CBP. IFN-γ-induced STAT1 binds to p300/CBP with very high affinity and thus blocks the AP1-mediated activation. Both in primary culture of skin fibroblasts and lung fibroblasts, IFN-γ-induced STAT1 is able to abrogate the TGF-β-induced collagen synthesis, and thus specific activation of STAT1 may be a useful approach in reducing the collagen synthesis in fibrotic skin or lung (111, 152).

TNF-α versus TGF-β. The sequences within the −265 to −241-bp of human COL1A2 promoter contain AP1 and NF-κB binding sites and are essential for TGF-β-mediated stimulation and TNF-α-mediated inhibition of COL1A2 promoter activity, and also for the antagonistic action of TNF-α on TGF-β stimulation. AP1 is a potential binding factor in this 25-bp element, and mutation in this region causes inhibition of basal as well as TGF-β-stimulated COL1A2 promoter activity. Further, overexpression of c-Jun inhibits the basal as well as TGF-β-induced COL1A2 promoter activity. As TGF-β induces the level of Jun-B and TNF-α induces the c-jun in dermal fibroblasts, authors implicated the c-Jun in TNF-α and TGF-β antagonistic action (109). The same group later showed that TNF-α inhibits the Smad-mediated TGF-β signaling by inducing c-Jun and Jun-B, which interact with Smad3 and inhibit the binding of Smad3 to the Smad binding element. Antisense c-Jun mRNA expression blocks the TNF-α-mediated inhibition of Smad-dependent TGF-β-induced transcription. Jun competes with Smad3 for limiting amounts of cellular p300/CBP transcriptional coactivators, suggesting that the antagonistic effect of TNF-α on Smad-dependent TGF-β signaling is mediated through induction of AP1. TNF-α does not induce the Smad7 expression in human dermal fibroblasts, and overexpression of dominant negative form of Iκ-B kinase-α or antisense Smad7 does not block the TNF-α-mediated inhibition of Smad-dependent TGF-β signaling, indicating that NF-κB and Smad7 are not involved in antagonistic action of TNF-α on TGF-β signaling (155). On the other hand, Bitzer *et al.* (156) reported that in mouse fibroblasts, NF-κB is required for TNF-α-mediated inhibition of Smad-dependent TGF-β signaling. NF-κB induces the level of anti-Smad, Smad7, in the mouse

embryonic fibroblasts, which blocks the TGF-β-induced phosphorylation, nuclear translocation, and DNA binding of Smads and thus blocks transcription from SBE reporter constructs. It is apparent that involvement of different factors in the antagonistic action of TNF-α and TGF-β on Type I collagen gene expression is cell type and promoter specific.

Concluding Remarks

Elevated level of Type I collagen in scleroderma skin fibroblasts is primarily due to the increased rate of collagen gene transcription (4, 6–8, 157). Extensive studies have been made to characterize the regulatory elements and transcription factors that regulate the Type I collagen gene expression under normal physiological condition and upon cytokine modulation, but so far, very little information has been gathered in the context of their implications in scleroderma or other tissue fibrosis. Increasing evidence suggests that TGF-β plays a significant role in fibrosis. Using TGF-β-induced normal skin fibroblasts as a model system for scleroderma skin fibroblasts, one can anticipate the possible mechanisms of the enhanced synthesis of collagens in human scleroderma skin fibroblasts. The possibilities are: (i) increased levels or activities of protein factors like CBF (39), Sp1 and NF1 (27), Smads signaling molecules or transcriptional coactivators p300/CBP (66), PN-1 (88), TGF-β and TGF-β receptors (158), and CTGF (98) or (ii) decreased level or activity of anti Smad, Smad-7, or unknown repressor that controls the magnitude of TGF-β- or Smad/p300-mediated induction of collagen synthesis, in scleroderma skin fibroblasts. It will also be very interesting and important to study the role of different known positive and negative modulators of the TGF-β signaling pathway in Type I collagen gene expression in response to TGF-β. Identification of positive or negative modulators of TGF-β-induced collagen gene expression and characterization of these modulators in scleroderma skin fibroblasts will be helpful to better understand the molecular causes of hypertranscription of collagen gene in fibrotic tissues.

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