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REVIEW ARTICLE

Molecular pathogenesis of fibrosis in systemic sclerosis

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ABSTRACT

The principal cause of fibrosis in systemic sclerosis is thought to be excessive deposition of extracellular matrix in multiple organs. The main component of matrix is thought to be collagen, especially type I collagen, which is one of the most abundant proteins in the mammalian body. Various factors have been estimated to be involved in the mechanism of their excessive deposition in fibrotic tissues of systemic sclerosis. In this review, we discuss the latest findings on these factors.

Keywords: Fibroblasts; Myofibroblasts; Collagen

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1. Introduction

The principal cause of fibrosis in systemic sclerosis (SSc) is assumed to be excessive deposition of extracellular matrix (ECM) in various organs. The main component of ECM is thought to be collagen, especially type I collagen, which is one of the most abundant proteins in the mammalian body.

Type I collagen molecule is a heterotrimer consisting of two α1(I) chains and one α2(I) chain, and the α1(I) and α2(I) collagen genes are located on chromosomes 17 and 7, respectively. Various factors, detailed below, have been estimated to be involved in the mechanism of their excessive deposition in SSc fibrotic tissues. In this review, we discuss the latest findings on these factors.

2. Fibroblasts

Since the ECM is mainly synthesized and secreted by fibroblasts during the fibrotic process, abnormalities of these cells have been thought to play a central role in pathogenesis of SSc. Fibroblasts belong to the mesenchymal lineage and are histopathologically identified as spindle-shaped cells. They have multiple origins, including bone marrow-derived progenitor cells, monocytes, epithelial cells, endothelial cells, and adipocytes, in addition to those resident in each tissue[1]. Fibroblasts are positive for a number of markers, including collagen, CD90, and FSP-1[2], but specific cellular markers have not been identified. These cells are activated in response to stimuli such as tissue injury and differentiate into myofibroblasts, which enhance ECM production. These myofibroblasts disappear due to regression or apoptosis as the wound epithelializes and stimulation decreases. In fibrotic lesions of SSc, however, the number of α-smooth muscle actin-positive

myofibroblasts is constantly increased[3–5], leading to excessive ECM deposition in various tissues and, ultimately, organ damage.

The mechanism of constitutive differentiation into myofibroblasts in SSc fibrotic tissue is thought to be complicated, and may include stimuli from the extracellular microenvironment, such as inflammation, immune abnormalities, and vascular abnormalities. Many studies have supported the concept, for example, CD301b-positive macrophages were shown to induce fibroblasts to differentiate into myofibroblasts in an in vivo skin model[6]. Conversely, fibroblasts derived from SSc fibrotic tissues continue to maintain their characteristics as activated myofibroblasts, even in an *in vitro* culture system isolated from such microenvironments[7].

2.1 Collagen gene promoter and transcription factors

The transcriptional activity of the α2(I) collagen gene is constantly up-regulated in cultured SSc dermal fibroblasts, and the responsible sequence is located in the -376 to -108 bp region of the promoter[7]. This region contains binding sites for transcription factors such as Sp1, Smad, and Ets family, and many changes in their expression, phosphorylation, and DNA binding ability have been reported, as described below. For example, in cultured fibroblasts derived from SSc lesional skin, the serine residue of Sp1 is constantly phosphorylated[7]. Furthermore, Sp1-specific inhibition with mithramycin suppresses the increased promoter activity of α2(I) collagen genes in these cells.

Smad is a well-known downstream mediator of transforming growth factor (TGF)-β signaling. TGF-β binding to its receptor induces the phosphorylation of Smad2 and Smad3, which in turn forms a complex with Smad4 and translocates into the nucleus. The complex binds to the CAGA motif, a Smad-binding sequence, on various promoters including α1(I) collagen and α2(I) collagen, and it regulates transcriptional activity by interacting with other transcription factors, such as the co-activators p300/CBP and Sp1[8]. Both activation and inhibition of Smad may promote tissue fibrosis depending on the circumstances, and although the detailed mechanism is not fully understood, the suppression of skin and lung fibrosis in Smad3 knockout mice suggests its involvement in the fibrotic process of multiple organs of patients with SSc[9]. While abnormal expression and phosphorylation of Smad3 have been reported in cultured SSc dermal fibroblasts, we have also demonstrated that phosphorylation and nuclear translocation of Smad2 and Smad3, as well as their interaction with Sp1, p300/CBP, and the α2(I) collagen gene promoter region, are constantly enhanced (without exogenous TGF-β stimulation)[7,10].

The Ets family includes more than 30 transcription factors that share a common structure, the Ets domain. Through that domain, they bind to Ets binding sequences including GGAA/T of various gene promoters. The expression of Fli1, which represses α2(I) collagen promoter activity, is decreased in cultured SSc dermal fibroblasts[11], while the DNA-binding capacity of Ets1, which stimulates the promoter activity, is constitutively increased, suggesting that there is switching between Ets1 and Fli1 at the Ets binding site on the α2(I) promoters[7].

c-Myb promotes the expression of α1(I) and α2(I) collagen genes in normal dermal fibroblasts[7], and is constantly up-regulated in SSc skin-derived fibroblasts.

AP-1 is composed of c-Fos, c-Jun, ATF, and JDF family members. Among them, c-Jun is usually expressed at very low level, but is up-regulated in fibrotic lesions[12]. Fra-2 belongs to the Fos family, and is up-regulated in SSc lesional skin[13]. Fra-2 is thought to induce apoptosis in vascular endothelial cells, and to stimulate collagen expression in dermal fibroblasts.

KLF5 is a member of the Krüppel-like family of transcription factors. It induces fibrotic process in cardiac fibroblasts and inhibits fibrosis in renal tubular cells[14,15]. On the other hand, KLF5 expression is suppressed in SSc lesional skin and cultured SSc dermal fibroblasts[16]. It is also a potent transcriptional repressor of CTGF, as described below[11].

2.2 Signaling pathways

In terms of JAK-STAT pathway, inhibition of STAT3 *in vitro* suppresses the TGF-β-induced differentiation of lung fibroblasts into myofibroblasts and the TGF-β-induced fibrosis-related gene expression[17]. Furthermore, genetic polymorphism of STAT4 is also known to be associated with the development of SSc[18].

Toll-like receptor (TLR) 4-responsive gene signatures are enhanced in biopsy specimens of SSc skin[19]. Activation of TLR4 signaling in cultured SSc fibroblasts resulted in enhanced collagen synthesis and increased expression of fibrosis-related genes[20]. Alternative spliced tenascin-C and fibronectin-EDA are considered to be its ligand. TLR4 also enhances the sensitivity of fibroblasts to the stimulatory effect of TGF-β1. In addition, we have shown that expression of TLR9, TLR5 or TLR10 is also increased in the skin or cultured dermal fibroblasts of patients with SSc[21,22].

2.3 Cytokines/chemokines

TGF-β, especially TGF-β1, is a key cytokine for the fibrogenesis in SSc. The cytokine promotes the differentiation of fibroblasts into myofibroblasts, and strongly induces collagen expression. In cultured fibroblasts derived from skin lesions of patients with SSc, there is thought to be constitutive activation of TGF-β signaling pathway, based on previous reports indicating that (1) inhibition of the TGF-β pathway suppresses the abnormal increase in α2(I) collagen gene expression, (2) α2(I) collagen gene expression is up-regulated while responsiveness to exogenous TGF-β stimulation is down-regulated, and (3), the sequence responsible for constitutive transcriptional activation of the α2(I) collagen gene promoter is identical to the TGF-β-response region of the promoter in normal dermal fibroblasts[7]. Nevertheless, the levels of secreted TGF-β were not different between culture media of normal and SSc dermal fibroblasts[23]. Ihn *et al.* hypothesized that TGF-β signaling is endogenously activated in SSc dermal fibroblasts, even in the absence of TGF-β (autocrine TGF-β signaling hypothesis)[24]. As its mechanism, the expression of TGF-β receptor is up-regulated in SSc fibroblasts[7]. Another mechanism is that TGF-β is secreted into the extracellular space as a small latent complex (SLC) by binding with latent TGF-β binding protein-1 (LTBP-1) and latency-associated peptide (LAP). In this SLC state, TGF-β cannot bind to its receptor, but the cytokine recovers binding activity when LAP is degraded by proteases such as plasmin or thrombin, or when TGF-β is dissociated by physical interaction between LAP and integrin or thrombospondin-1[7]. We found that integrins on the cell surface of SSc dermal fibroblasts are overexpressed, and activate TGF-β signaling by activating SLC without increasing the amount of TGF-β[25]. Thrombospondin-1 is also up-regulated in SSc dermal fibroblasts, and may play a role in the activation of TGF-β signaling[7]. On the other hand, failure of negative feedback mechanisms, such as Smad7, may also be one of the reasons why abnormal TGF-β activation is maintained in SSc dermal fibroblasts[10]. Similarly, c-Ski/SnoN expression is also increased in SSc dermal fibroblasts, but its function of antagonizing p300 was found to be impaired[7]. The abnormal activations of TGF-β in SSc fibroblasts can explain many changes, such as increased phosphorylation or DNA binding of Smad and its interaction with p300/CBP, activation of Ets1 DNA binding, down-regulation of Fli1, and abnormal TLR expression as described above[7,21,22]. In addition, a number of downstream molecules mediate the effects of TGF-β, such as c-Myb, STAT3, Wnt-3a, and PTP4A1[7,26,27]. Moreover, TGF-β inhibits the production and secretion of matrix metalloproteinase (MMP) from dermal fibroblasts, and induces the expression of tissue inhibitor of metalloproteinase (TIMP), an inhibitor of MMP. These changes strongly suppress collagen degradation, thereby enhancing excessive collagen deposition in tissues and further contributing to fibrosis in this disease[7].

Connective tissue growth factor (CTGF) is induced by TGF-β as a downstream target, and promotes the production of ECM through various mechanisms, such as indirect regulation of Smad in collaboration with TGF-β. In addition, CTFG plays a role in the differentiation of endothelial cells into myofibroblasts[4]. Subcutaneous injection of TGF-β

alone transiently induces fibrosis in mice skin, whereas the subsequent addition of CTGF sustains the fibrosis[7]. TGF-β may therefore induce initial fibrosis of SSc, which may be maintained by CTGF (two-step fibrosis hypothesis)[28]. In serum, skin, and cultured dermal fibroblasts from patients with SSc, the levels of CTGF are increased in proportion to the degree of fibrosis[7]. In addition, a genetic polymorphism at the Sp1/3 binding site on the CTGF promoter is reported to be associated with the increased CTGF levels in patients with SSc[29].

The interleukin (IL)-1 superfamily includes IL-1α, IL-1β, and IL-1 receptor antagonist, and expression of inflammasome gene transcripts are increased in SSc dermal and lung fibroblasts, reflecting the activation of this pathway[30]. IL-1α stimulates collagen production and cell proliferation in normal fibroblasts by inducing the expression of IL-6 and platelet-derived growth factor (PDGF)-A, as described below. Furthermore, endogenous IL-1α levels are increased in cultured SSc dermal fibroblasts[31], and inhibition of IL-1α suppresses the expression of IL-6 and collagen.

The amino acid sequences of IL-4 and IL-13 show about 30% homology, and their receptors also share common components. Both IL-4 and IL-13 are up-regulated in the skin tissue and serum of patients with SSc, and protein expression of IL-4 in particular is also increased in cultured SSc dermal fibroblasts[7,32]. IL-4 and IL-13 induce TGF-β-independent up-regulation of type I collagen to the same extent in cultured normal dermal fibroblasts[33–35], while only IL-4 stimulates TIMP-2 expression and further promotes fibrosis. In contrast, IL-13-deficient mice are less likely to develop pulmonary fibrosis due to suppressed collagen production from fibroblasts, whereas IL-4-deficient mice do not show such effect[7]. The roles of IL-4 and IL-13 in fibrosis may therefore be different *in vitro* and *in vivo*.

IL-6 has been reported to be increased in the serum and skin of patients with SSc. It acts on myofibroblasts to induce the expression of type I collagen via amplification of the TGFβ-Smad3 signaling pathway[36–38].

IL-17 may both inhibit and induce collagen expression *in vitro*, but it is thought to be pro-fibrotic at least *in vivo*[39,40].

Endothelin-1 is a molecule associated with the formation and function of blood vessels, and its serum levels are elevated in patients with SSc. It induces fibroblast differentiation into myofibroblasts, and stimulates expression of type I collagen and CTGF. On the other hand, endothelin-1 also suppresses the expression and activity of MMP-1, which may contribute to maintaining excessive collagen deposition[7,41,42].

Angiotensin II expression is increased in SSc serum and skin, and its type 2 receptor is also up-regulated in SSc fibroblasts[43]. Its action is thought to induce type I collagen expression in fibroblasts[7].

Expression of PDGF and its receptor (PDGFR) is increased in SSc skin and fibroblasts[44]. In addition, PDGFR-stimulating antibodies are detected in the sera of patients with SSc; they induce type I collagen expression in fibroblasts and promote differentiation into myofibroblasts[45].

As in lupus erythematosus and other autoimmune diseases, activation of type I interferon signature and gene mutations of interferon-related genes have been found in some patients with SSc[46,47]. In lung lesions of patients with SSc, disease activity correlates with expression of the interferon-related genes[48].

Vascular endothelial growth factor (VEGF) production in SSc dermal fibroblasts is increased by the stimulation of autocrine TGF-β/Smad signaling[49]. VEGF can directly induce collagen synthesis in dermal fibroblasts[50].

In fibroblasts, activation of Wnt/β-catenin signaling, a pathway involved in tissue development, stimulates cell proliferation and migration, expression of fibrosis-related genes, and differentiation into myofibroblasts[51]. In SSc skin tissue, the amounts of nuclear β-catenin, the Wnt signaling receptor FZD2, and its downstream target LEF1 are reported to be increased, while those of Wnt antagonist DKK2 and WIF1 are decreased[52]. Wnt signaling may therefore also play a role in fibrosis in this disease.

In addition, sonic hedgehog, which is also in-

volved in tissue development, is strongly expressed in fibroblasts, keratinocytes and endothelial cells of SSc skin, and induces differentiation into myofibroblasts and increases collagen production[53]. The involvement of the Notch pathway and the YAP/TAZ/Hippo pathway has also been shown in other studies.

Among chemokines, CX3CL1 is a fractalkine that functions as chemotactic factor and adhesion molecule. Expression of its receptor, CX3CR1, has been found in a variety of cells. Hasegawa *et al.* showed that increased expression of CX3CL1 in skin and serum was observed in a bleomycin-induced fibrosis in mice model[54,55]. Its neutralizing antibody suppressed fibrosis induced by subcutaneous injection of TGF-β and CTGF, suggesting that CX3CL1 mediates the fibrotic effects of these cytokines.

3. Involvement of other cells

In the natural history of this disease, autoimmunity, inflammation and vascular damage precede the appearance of fibrosis. Other cells are therefore assumed to be involved in the fibrotic process through a variety of mechanisms, including the following.

3.1 Innate immunity

M2 macrophages and CD301b-positive macrophages as well as mast cells and plasmacytoid dendritic cells are thought to play a role in tissue fibrosis via effectors such as TGF-β, IL-1, IL-6, and TLRs[2,6,56].

3.2 Acquired immunity

Patients with SSc have increased numbers of activated B cells in the blood, which activate fibroblasts through the secretion of cytokines such as IL-6 and TGF-β[57]. The production of autoantibodies against fibroblast, fibrillin, PDGFR, and matrix metalloproteinase can also directly induce fibrosis[58]. In addition, abnormality of regulatory B cells, which suppress excessive immune response and inflammation through IL-10 production, was proved in patients with SSc[59]. Furthermore, CD8-positive T cells, Th1, Th17, and innate lymphocytes also contribute to the pathogenesis through various cytokines and signaling pathways including JAK-STAT pathway[2,60].

3.3 Vascular system

In patients with SSc, vascular damage, ischemia-reperfusion injury associated with Raynaud's phenomenon, generation of reactive oxygen species, and tissue hypoxia occur in multiple organs. Hypoxia induces cytokines such as TGF-β and hypoxia-inducible factors that cause mesenchymal cells to differentiate into myofibroblasts. In addition, vascular endothelial cells, platelets, and pericytes have also been implicated in tissue fibrosis.

3.4 Epithelial cells

Epidermal cells in SSc lesional skin are involved in fibrosis through cytokine secretion[61]. Furthermore, for example, Fli-1 deficiency in keratinocytes induces skin fibrosis in mice model[1].

4. Epigenetics

4.1 DNA methylation and histone modification

The methylation status of many genes is altered in SSc dermal fibroblasts. Methylation levels of ITGA9, ADAM12, COL23A1, COL4A2, MYO1E, and RUNX family members are reduced, for example, and their gene overexpression tends to be up-regulated[62].

In addition, because collagen expression in SSc dermal fibroblasts is reduced by trichostatin A, histone deacetylase inhibitor, histone modification also contributes to the pathogenesis of excess collagen production[63]. Expressions of histone demethylases such as MeCP2, JMJD3, and SIRT1 are changed, which regulate the expression of various collagen-related molecules including P300[64–66].

Additionally, in SSc dermal fibroblasts, methylation and histone deacetylation of promoters cause the decrease in Fli1 and KLF5[11]. Trimethylation of histone H3 on lysine 27 (H3K27me3) also controls the expression of Fra-2[67].

4.2 MicroRNAs

Maurer *et al.* demonstrated that

down-regulation of miR-29a, -29b, and -29c in SSc dermal fibroblasts causes an increase in their targets, α1(I) and α2(I) collagen[68]. In addition, we found that miR-196a and let-7a also target collagen, and their expression is decreased in SSc dermal fibroblasts by autocrine TGF-β signaling[69]. Furthermore, we reported that decreased expression of miR-150 or increased expression of miR-92a in these cells may lead to the increased expression of integrin β3 or decreased expression of MMP-1, respectively[69,70]. Multiple microRNAs may also therefore be involved in the process of abnormal collagen expression in SSc fibroblasts and organ fibrosis.

5. Conclusion

The mechanism of tissue fibrosis in SSc has not yet been completely elucidated, but various researches have continued to prove the contribution of many factors to the mechanism. The involvement of adipocytes and metabolic pathways has also been described, and further research is expected to identify new immunotherapeutic targets.

Conflict of interest

The author declares no conflict of interest.

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