What did we learn by studying scleroderma fibroblasts ?

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ABSTRACT

Systemic sclerosis (SSc, scleroderma) is a complex disease manifesting itself by fibrosis of skin and other internal organs. Fibroblasts isolated from SSc lesions and cultured in vitro *are characterized by the increased synthesis of collagen and other extracellular matrix (ECM) proteins, consistent with the disease phenotype. Cultured SSc fibroblasts therefore serve as a principal experimental model for studying the molecular and cellular mechanisms involved in collagen overproduction in this disease. This article provides an overview of transcription factors that are deregulated in SSc fibroblasts. The possible origin of SSc fibroblasts is also discussed.*

Introduction

When Dr. E. Carwile LeRoy unexpectedly passed away in 2002 after attending a scientific meeting in Italy, the scleroderma community lost one of its most influential and supportive members. Dr. LeRoy, well known and admired around the world as one of the pioneers of scleroderma research, had devoted his entire career to unraveling this complex disease. I was privileged to work with Carwile for a number of years and would like to dedicate this article to his memory.

Scleroderma fibroblast takes center stage

Over 30 years ago, in 1972, LeRoy published his seminal observation that fibroblasts from the skin of patients with scleroderma, when isolated and propagated *in vitro*, produce more collagen than fibroblasts isolated from the skin of healthy people (1). This was based on previous work by Rodnan and Fleischmayer showing that the increased collagen protein deposited in the skin of scleroderma patients gives the skin its characteristic tight and hidebound appearance (2-4). LeRoy's observation had several important implications. First, it suggested that abnormally activated fibroblasts present in the skin of scleroderma patients are responsible for the augmented deposition of collagen in the skin. Because scleroderma fibroblasts remained activated during *in vitro* culturing, it suggested that this defect was an intrinsic property of these cells. Importantly, it was now possible to study this defect in the laboratory and to investigate the physiology that caused the activated state of scleroderma cells. Indeed, LeRoy's publication has inspired a number of researchers around the world, including our laboratory in Charleston, to pursue these questions. I would like to take this opportunity to describe the progress that has been made in understanding activation of scleroderma fibroblasts and discuss the challenges that still remain.

Biosynthetic pathways are altered in scleroderma fibroblasts

In the years that followed LeRoy's publication the production levels of several other proteins by scleroderma fibroblasts were found to be altered. Some are produced at higher levels and others at lower levels. Scleroderma fibroblasts were shown to produce elevated levels of other extracellular matrix (ECM) components, including collagens type I, III, VI, VII, XVI, fibronectin, decorin and glycosoaminoglycans. Other proteins including growth factors such as IL-6, pro-IL-1α, CTGF, cell surface receptors such as ICAM-1, and both signaling molecules and transcription factors are also synthesized at higher levels [reviewed in (5-8)]. Taken together these studies have shown that scleroderma fibroblasts exhibit alterations in the regulatory pathways that control ECM production and other cellular responses linked to the process of fibrosis. Because of technological advances we may soon know most of the proteins which are differentially produced by scleroderma fibroblasts. Recent sequencing of the human genome has given

rise to new technologies, including DNA microarrays and proteomics that have revolutionized the way in which gene expression is analyzed. Several scleroderma investigators are currently applying these new methods to catalogue the mRNAs and proteins differentially expressed in scleroderma fibroblasts. These studies suggest that several hundred mRNAs may be differently expressed. Although verification of these differences will take some time, once this task is finished the description of how scleroderma fibroblasts differ from healthy fibroblasts, which originated with LeRoy's observation, will be completed.

Altered balance of transcription activators and repressors is responsible for deregulation of collagen gene in scleroderma

Cultured scleroderma fibroblasts became an experimental model system utilized by many investigators to uncover the molecular and/or cellular mechanisms responsible for their activation. Regulation of collagen type I gene expression became a primary focus of these investigations. The collective efforts of several laboratories have established that scleroderma fibroblasts, as a result of an elevated transcription rate (9) and increased mRNA stability (10), exhibit increased steady-state levels of collagen type I mRNA (11, 12). Subsequent work by Kikuchi *et al.* (13) and Hitraya *et al.* (14) demonstrating the increased promoter activities of the collagen α2(I) (COL1A2) and collagen α1(I) (COL1A1) genes provided the basis for systematic analyses of the transcriptional mechanisms involved in deregulation of the collagen type I genes.

There has been steady progress in delineating these mechanisms and several transcription factors with altered expression levels or post-translational modification status in scleroderma fibroblasts have been described [recently reviewed in (15-18)]. The significance of each of these factors in the pathogenesis of scleroderma needs to be validated in patients *in vivo* and, if possible, in an animal model of scleroderma. Such data have been recently generated for Smad3 and the repressor of collagen gene, Fli1 (see below). Transcription factors with possible roles in scleroderma, representing both transcription activators and transcription repressors, are shown in Figure 1 and briefly described below.

Sp1

Sp1 is a ubiquitously expressed zinc finger transcription factor implicated in the regulation of numerous viral and cellular genes (19). Sp1 is a major positive regulator of the basal expression of both COL1A1 and COL1A2 genes. In cooperation with other transcription factors, Sp1 is also involved in mediating intracellular signaling pathways including those of TGF-β (20), OSM (21), serum (22), and TNF-α (23). Scleroderma and control healthy fibroblasts express similar levels of Sp1 protein. However, Sp1 is more highly phosphorylated in scleroderma fibroblasts (24). Furthermore, increased Sp1 phosphorylation correlates with increased levels of COL1A2 mRNA.

CBF/NF-Y

CBF/NF-Y is a ubiquitously expressed heterotrimeric CCAAT binding factor, which regulates the basal expression of COL1A1 and COL1A2 genes (25, 26). Interestingly, recently published studies suggest that CBF/NF-Y mediates mechanical strain induction of the COL1A1 promoter via a TGF-β-dependent pathway (27). Given the known tissue- and species-specific differences involved in regulation of collagen genes (28) (the latter studies were performed using rat cardiac fibroblasts and rat COL1A1 promoter) these observations require verification in human dermal fibroblasts. Nonetheless, these finding are of great interest since previous studies have shown that dermal fibroblasts subjected to the mechanical stress display a "synthetic" phenotype (29). Increased binding of CBF/NF-Y to the proximal CCAAT box in the COL1A1 promoter has been observed in scleroderma fibroblasts (30).

C-myb

C-myb expression is induced by TGF-β in dermal fibroblasts, and an elevated expression of c-myb has been demonstrated in unstimulated scleroderma fibroblasts (31, 32). C-myb regulates basal expression of the human COL1A2 promoter and, although its expression is stimulated by TGF-β, it is not required for the TGF-β upregulation of the collagen type I gene (33).

Smads

Smads are downstream mediators of the TGF-β signaling pathway (34). Smad3 has been shown to mediate the induction of COL1A2 (35, 36) and CTGF (37) in healthy dermal fibroblasts. Increased Smad2/3 phosphorylation levels and increased nuclear localization of Smad3 and Smad4 have been found in scleroderma fibroblasts (38, 39). In addition, activity of the Smad-responsive minimal promoter was elevated in scleroderma fibroblasts (38). Consistent with these data, elevated expression of Smad3, but not Smad2 was noted in scleroderma skin (39). In addition, Dong *et al.* reported a marked downregulation of an inhibitory Smad, Smad7, in scleroderma fibroblasts *in vitro* and in scleroderma skin *in vivo* (39). However, Smad7 alterations in scleroderma fibroblasts were not confirmed in a subsequent study by Mori *et al.* (38). While further clarification is needed regarding Smad7 deregulation in scleroderma, together these studies strongly suggest that alterations in the Smad pathway contribute to ECM deregulation in scleroderma.

Fli1

Fli1, a member of the evolutionary conserved Ets1 family of transcription factors, is required for megakaryocytic differentiation and vascular remodeling during embryonic development (40). Studies using cultured dermal fibroblasts have shown that Fli1 suppresses ECM synthesis (41). More importantly, our recent studies have demonstrated that Fli1 is prominently expressed in skin fibroblasts *in vivo* and may play an important role in the regulation of collagen homeostasis in human skin (42). That the expression of Fli1 is consistently reduced in scleroderma skin may directly contribute to the activation of scleroderma fibroblasts and uncontrol-

led matrix deposition (42). The importance of Fli1 in the pathogenesis of scleroderma is further supported by studies using a murine genetic model. The targeted deletion of the Fli1 gene is homozygous lethal at 11.5 dpc (43). Heterozygous $Fli1^{+/-}$ mice are viable and produce more collagen in the skin, which correlates with an increased number of fibroblasts expressing COL-1A1 mRNA as compared to wild type mice (M. Kubo, M. Markiewicz, and M. Trojanowska, unpublished observations). Analyses of mouse embryonic fibroblasts (MEFs) obtained from $Fli1^{-/-}$, $Fli1^{+/-}$, and $Fli1^{+/+}$ mice indicate that the absence of Fli1 leads to a large increase in collagen type I mRNA and protein levels as compared to the wild type fibroblasts, while $Fli1^{+/-}$ MEFs exhibit an intermediate phenotype (42). These observations suggest that the downregulation of Fli1 is a critical pathogenic event leading to deregulated collagen production in scleroderma.

In healthy tissue fibroblasts, the balance between co-activators and co-repressors interacting with basal transcription factors, e.g. Sp1, determines the transcription activity of collagen type I gene. Currently available data strongly suggest that this balance is altered in scleroderma. As briefly discussed in this section, some of the transcription factors that are expressed at altered levels in scleroderma are already known and additional factors undoubtedly will be discovered. Since transcription factors are the final effectors of the signaling pathways, the important next step will be to link specific pathways to transcription factors that contribute to the scleroderma phenotype. One such pathway may be mediated by PKCδ, which has been shown to be elevated in scleroderma fibroblasts (44), but its connection to putative transcription effectors has not been yet characterized. Thus, although significant progress has been made in understanding the molecular components that contribute to scleroderma, much work still remains to be done before a comprehensive view of the cellular and molecular mechanisms responsible for the activation of scleroderma fibroblasts will be achieved.

What causes the switch of scleroderma fibroblast to activated state remains unknown

Numerous *in vivo* studies indicate that scleroderma fibroblasts acquire their activated status very early during disease progression (45), but the underlying mechanisms responsible for the switch to activated status remain unknown. One possible scenario is that other cells present in the scleroderma lesion during this early stage, e.g. inflammatory cells, produce the signals that either "activate" the resident fibroblasts or selectively amplify the subpopulation of fibroblasts with the pre-existing activated phenotype (6, 46). TGF-β, acting in concert with other cytokines or chemokines secreted by the activated immune cells, may constitute such signals (47). In addition, cell-cell contact between immune cells and fibroblasts may play a role in fibroblast activation and/or selection (48). Another possibility is the transdifferentiation of other cell types, e.g. pericytes, into activated fibroblasts. It is well known that degeneration of the small blood vessels is one of the earliest manifestation of the disease (49, 50). The fate of the pericytes associated with such degenerating blood vessels is not clear. Activation of microvascular pericytes has been reported in early scleroderma and autoimmune Raynaud's phenomenon (51). In scleroderma lesions, pericytes have been characterized by the expression of PDGFβ receptors and HMW-MAA (high molecular weight melanoma-associated antigen), a marker for activated pericytes. Interestingly, previous studies in dermal scarring have shown that, *in vivo*, microvascular pericytes that express both HMW-MAA and PDGFβ receptors can migrate from the microvasculature and undergo a gradual phenotypical transition to collagen-synthesizing fibroblasts (52), suggesting that fibroblasts isolated from scleroderma lesions may contain a proportion of pericyte-derived collagen producing cells. Further studies are needed to determine whether there are similarities between the behavior of transdifferentiated pericytes and scleroderma fibroblasts.

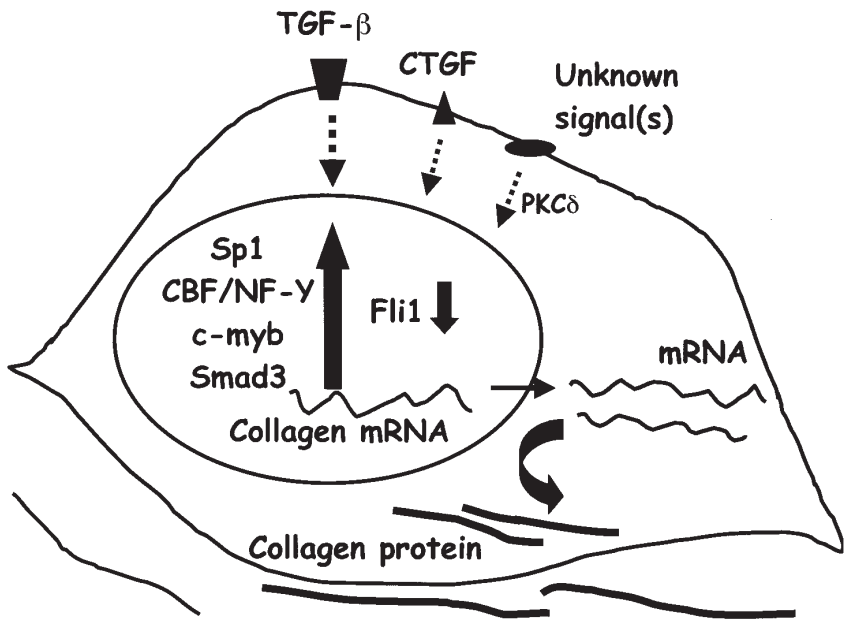


Fig. 1. Activation of scleroderma fibroblasts results from the altered balance of transcription activators and repressors. With regard to collagen type I transcription, activators with elevated levels or activities include Sp1, CBF/NF-Y, c-myb, and Smad3, while a suppressor, Fli1, is reduced in scleroderma fibroblasts. The signal transduction pathways that regulate formation of the specific transcription complexes in healthy skin fibroblasts and are deregulated in scleroderma fibroblasts are still poorly defined.

Conclusions

A successful treatment for scleroderma remains a challenge. Three decades after Dr. LeRoy's initial observations the

hope remains that full knowledge of the regulatory circuits that are altered in scleroderma will ultimately lead to novel therapies. Based on such knowledge the key molecules can be selectively targeted to prevent or reverse the progress of fibrosis. Because of the accelerated pace of scientific discoveries taking place today we should be optimistic that the remaining questions will be answered in the near future, to the benefit of scleroderma patients.

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